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LEPROSY IN SQUIRRELS:

AN ANCIENT DISEASE IN AN ENDANGERED WILDLIFE HOST

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Declaration

I declare that this thesis has been composed by myself, and that my input to the disease investigations was as described in the Materials and Methods. I confirm that the work submitted is my own, except where work which has formed part of jointly authored publications has been included. My contribution and those of the other authors to this work have been explicitly indicated below. Some of the findings have been previously published in letters, short communications or papers in the scientific press, as referenced in the appropriate sections of the thesis. The body of work presented in this thesis has not been submitted for any other degree or professional qualification.

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Anna-Katarina Schilling

23/08/2019

Abstract

Leprosy is an ancient human disease that was thought to have been eradicated from the British Isles. The last case of autochthonous human infection was documented in the 1950's. Natural infection with leprosy bacilli in species other than humans was first described in nine-banded armadillos in the 1970s in the United States of America. Recently, both bacterial species causing leprosy, *Mycobacterium leprae* and *Mycobacterium lepromatosis*, were isolated from Eurasian red squirrels (*Sciurus vulgaris*, ERS) across the British Isles. ERS are endangered in this part of their range, and efforts are made for their protection.

This thesis offers insight into important aspects (clinical presentation, pathology, epidemiology) of the basic description of leprosy in live ERS, based on data from two wild British island ERS populations naturally infected with leprosy bacilli. The populations, one in Scotland and one in England, were studied for 18 and 24 months respectively, with live sampling taking place every six months. Additionally, samples from ERS, Eastern grey squirrels (*Sciurus carolinensis*, GS) and Pallas's squirrels (*Callosciurus erythraeus*, PS) were obtained from Britain (ERS, GS), Germany (ERS, PS) and Italy (ERS, GS, PS) and screened for the presence of leprosy bacilli to provide new epidemiological surveillance information on squirrel leprosy.

Established, adapted, and novel tests were used to diagnose leprosy in squirrels. Accurate clinical diagnosis is important to identify populations affected by the disease. Serological methods were useful to confirm the clinical diagnosis. Molecular methods were the only way to identify leprosy bacilli in squirrels without clinical signs of disease. A diagnostic decision tree is proposed to allow optimised, consistent use of the methods now available depending on the situation in which a diagnosis is sought.

ERS that are infected with *M. leprae* and develop clinical leprosy usually showed a multibacillary, lepromatous or borderline lepromatous form of the disease. Lepromatous leprosy is characterised by an inability of the host immune response to control bacterial replication and dissemination. Leprosy in ERS progressed slowly, and the intensity of lesions could easily be separated into four categories from mild to severe based on lesion size, structural characteristics and the presence or absence of ulceration. Several months passed between the time when the bacteria first became detectable in an ERS tissues and the onset of clinical disease. Clinical disease then progressed on varying timescales in different individuals, but usually allowed the individuals to thrive for long time frames (months – years). The maximum time period a clinically diseased ERS was followed in this study was 18 months. Prevalence and morbidity differed in individual ERS populations. In one population the total apparent two-year prevalence of leprosy was 36% with a morbidity rate of 22% for the same population and timeframe. In the other the apparent two-year prevalence was only 4% and no clinical cases of leprosy were observed. The presence of leprosy did not have a negative effect on individual ERS or whole populations that could be measured using health indicators such as body condition, weight, general health and ectoparasite burdens. As part of this study, *M. leprae* was identified in ERS in two new locations within the UK, but not in British GS or any squirrel species in Germany or Italy.

The results indicate that leprosy alone is unlikely to be a major factor contributing to ERS mortalities and thus may not be of great conservation concern in this species. Continued research into ERS leprosy in natural systems could provide valuable insight into disease dynamics that may benefit humans and other hosts in a One Health and conservation medicine framework.

Lay summary

Leprosy is an ancient human disease that no longer occurs in people in the British Isles, other than for a few occasional cases in which the patients became infected abroad. Two bacteria, *Mycobacterium leprae* and *Mycobacterium lepromatosis*, cause the disease. It has only been known since the 1970's that leprosy bacilli can also cause disease in other species. Then the bacilli were found in nine-banded armadillos in the United States of America. Within the last 5 years it was discovered that skin lesions in Eurasian red squirrels (ERS) in the British Isles can be caused by leprosy bacilli. ERS are endangered in this part of their range and efforts are made to protect them.

This thesis provides insights into the effect leprosy is having on ERS on an individual and population level, and also explores whether the disease is limited to ERS in the British Isles. Two island ERS populations, one in England, one in Scotland, in which the presence of leprosy bacilli has been confirmed previously, were studied over a two-year period. Additionally, samples from ERS and other squirrel species were obtained from Britain, Germany and Italy and it was determined whether they contained leprosy bacilli. Methods that can be used in live and dead ERS to diagnose leprosy were assessed and adapted where necessary. The clinical diagnosis of leprosy is extremely important in ERS to identify where the disease occurs. Laboratory methods were useful to confirm the clinical diagnosis and to identify leprosy bacilli in ERS that do not (yet) show clinical signs of disease.

ERS infected with *M. leprae* usually showed a form of the disease in which large numbers of bacilli are present and the main clinical signs are hairless nodular swellings on ears and hocks. Leprosy progressed slowly in ERS, and several months passed between the first detection of bacilli and the onset of clinical lesions. Leprosy then progressed over varying timescales in individual ERS, but usually allowed affected ERS to thrive for months and sometimes years. The maximum time period a diseased ERS could be followed in this study was 18 months. The proportion of ERS affected by leprosy differed between the two populations. In one, leprosy bacilli were found in over a third of ERS seen within the two years of the study, with about 1/5 showing clinical signs of disease. In the other population no clinically diseased ERS were seen and leprosy bacilli were present in only 4% of the ERS assessed. As part of this study, *M. leprae* was identified in ERS in two new locations within the UK, but not in British grey squirrels or any squirrel species in Germany or Italy.

The results indicate that leprosy alone is not a threat to British ERS. Continued efforts are necessary to identify the full host range of leprosy bacilli around the world. Studying leprosy in this endangered wildlife host in the wild may offer a unique opportunity for future research: to understand the transmission of leprosy in a natural disease system. This study provides relevant tools and insights to form a basis for such efforts.

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List of Abbreviations

αPGL-I	Anti-*M. leprae* phenolic glycolipid-I IgM antibodies
aDNA	Ancient Deoxyribonucleic acid
AFB	Acid-fast bacilli
AR	Isle of Arran
BB	Borderline borderline leprosy
BCF	Body cavity fluid
BCS	Body condition score
BI	Brownsea Island
BIn	Bacterial Index
BL	Borderline lepromatous leprosy
BT	Borderline tuberculoid leprosy
CRP	C reactive protein
CPR	Cardiopulmonary resuscitation
DLL	Diffuse lepromatous leprosy
DNA	Deoxyribonucleic acid
Dr.	Doctor
ENL	Erythema nodosum leprosum
ERS	Eurasian red squirrel
FF	Fite-Faro stain
FS	Finlayson's squirrel
GHS	General health status
GS	Eastern grey squirrel
HSLF	High salt lateral flow buffer
IDRI	Infectious Disease Research Institute
InL	Indeterminate leprosy
IL	Interleukin
INF	Interferon
IUCN	International Union for Conservation of Nature
LFA	Lateral flow assay
LID-1	Leprosy IDRI diagnostic-1
LL	Lepromatous leprosy
M.	*Mycobacterium*
MB	Multibacillary
NBA	Nine-banded armadillo
PADL	protein advances for the diagnosis of leprosy
PB	Paucibacillary
PCR	Polymerase chain reaction

PNL	Primary neural leprosy
PS	Pallas's squirrel
qPCR	Quantitative polymerase chain reaction
RDSVS	Royal (Dick) School of Veterinary Studies
RR	Reversal reaction
SNP	single-nucleotide polymorphisms
SQPV	Squirrelpox virus
TLR-I	Toll-like receptor I
TNF	Tumour necrosis factor
TT	Tuberculoid leprosy
UCP	Up-converting phosphor
UK	United Kingdom
UoE	University of Edinburgh
USA	United States of America
WHO	World health organisation
ZN	Ziehl-Neelsen stain

List of Publications

1. **Schilling, A.-K.**, Avanzi, C., Ulrich, R. G., Busso, P., Pisanu, B., Ferrari, N., Romeo, C., Mazzamuto, M. V., McLuckie, J., Shuttleworth, C. M., Del-Pozo, J., Lurz, P. W. W., Escalante-Fuentes, W. G., Ocampo-Candiani, J., Vera-Cabrera, L., Stevenson, K., Chapuis, J.-L., Meredith, A. L., Cole, S. T. (2019) **British Red Squirrels Remain the Only Known Wild Rodent Host for Leprosy Bacilli.** Frontiers in Veterinary Science (6), Article 8. https://doi.org/10.3389/fvets.2019.00008
2. **Schilling, A.-K.**, van Hooij, A., Corstjens, P., Lurz, P. W. W., DelPozo, J., Stevenson, K., Meredith, A., Geluk, A. (2019) **Detection of humoral immunity to mycobacteria causing leprosy in Eurasian red squirrels (*Sciurus vulgaris*) using a quantitative rapid test.** European Journal of Wildlife Research, 65:49. https://doi.org/10.1007/s10344-019-1287-1
3. **Schilling, A.-K**., Del-Pozo, J., Lurz, P. W. W., Stevenson, K., Avanzi, C., Shuttleworth, C. M., Cole, S. T., Meredith, A. L. (2019) **Leprosy in red squirrels in the UK.** Veterinary Record, 184(13), pp. 416. http://dx.doi.org/10.1136/vr.l1385
4. Hardouin, E. A., Baltazar-Soares, M., **Schilling, A.-K.**, Butler, H., Garcia-Rodriguez, O., Crowley, E., Liang, W.-J., Meredith, A., Lurz, P. W., Forster, J., Kenward, R. E., Hodder, K. H. (2019) **Conservation of genetic uniqueness in remaining populations of red squirrels (*Sciurus vulgaris L.*) in the South of England.** Ecology and Evolution, 00:1-12. https://doi.org/10.1002/ece3.5233
5. Tió-Coma, M., Wijnands, T., Pierneef, L., **Schilling, A.-K.**, Alam, K., Roy, J. C., Faber, W. R., Menke, H., Pieters, T., Stevenson, K., Richardus, J. H., Geluk, A. (2019**) Detection of *Mycobacterium leprae* DNA in soil; Multiple needles in the haystack?** Scientific Reports, 9:3165. https://doi.org/10.1038/s41598-019-39746-6
6. Lurz, P. W. W., Krauze-Gryz, D., Gryz, J., Meredith, A., **Schilling, A.-K.**, Thain, C., Heller, E. (2017) **Invisible threats to native mammals – mercury levels in three Eurasian red squirrel populations.** Hystrix, the Italian Journal of Mammalogy. https://doi.org/10.4404/hystrix-28.2-12335

Chapter 1: Leprosy - an ancient disease and its hosts

1.1. Introduction

Upon hearing the word leprosy, almost every person immediately has some dramatic, often prejudice driven and inaccurate, images in their mind. These include severely disfigured people, whose limbs or facial annexes will fall off when touched, and that need to be avoided at all costs. As a result, the disease is still associated with severe stigma and negative reactions from the wider society (WHO, 2017). This has been summarised in the phrase "Leprosy impairs and society disables" (Kumar, Lambert and Lockwood, 2019). Those images that people have in their heads have been helpful in attracting attention, raising awareness and driving donations, but they are not representative of the disease, and ultimately, these misconceptions hinder the understanding and control of the disease (Monte *et al.*, 2017; Kumar, Lambert and Lockwood, 2019). Few individuals are aware that humans are no longer the only host for the disease and how limited our knowledge still is about even basic disease processes, such as transmission.

This study aims to provide a basic description of leprosy in its most recently discovered endangered wildlife host, the Eurasian red squirrel (*Sciurus vulgaris*, ERS). This information would ideally enable a rational debate about how the presence of a disease that was thought to be eradicated in most of Western Europe should be addressed in a conservation sensitive host species.

1.1.1. Leprosy, an ancient disease

Leprosy – as a disease of man – has been described convincingly in ancient texts since 1500 B.C. (Rojas-Espinosa and Lovik, 2001). Palaeopathological findings suggest the occurrence of leprosy as far back as 3780-3650 calculated BC in Hungary, 2500-1700 BC in the Indus Valley and in the 4th-3rd century BC in Italy (Donoghue, 2019). Even greater certainty about the fact that leprosy has indeed been present in the human population for a huge part of its history is provided by the isolation of ancient *Mycobacterium leprae* DNA from archaeological specimens. *M. leprae* aDNA has been isolated from human remains dating back to the 1st to 7th century AD in Palestine, Uzbekistan, Egypt, and Israel (Donoghue, 2019). The genetically most ancestral *M. leprae* strains have been isolated from Japan, China, Korea, the Marshall Islands and New Caledonia (Benjak *et al.*, 2018).

In Europe, leprosy occasionally occurred in the Roman period, but it was during medieval times that its prevalence drastically increased (Donoghue *et al.*, 2015). Genetic evidence supports the hypothesis that ancient Greek and Roman travelling routes connecting Europe, the Middle East, East Africa and South Asia contributed to the spread of leprosy (Benjak *et al.*, 2018).

The oldest *M. leprae* genome isolated from human remains in the United Kingdom dates back to 415+/- 545 calibrated AD (Schuenemann *et al.*, 2018). In medieval times, leprosy was highly endemic all across Europe as attested by the existence of ~19,000 leprosaria between the 12th and 15th century (Little, Farmer and Rosenwein, 2000). The decline of human leprosy in Europe began in the 15-16th century (Schuenemann *et al.*, 2018). It persisted in the Iberian Peninsula, the Mediterranean, the Balkans, Scandinavia and Baltic countries into the 20th century. About 10,000 human cases were recorded in Norway, Sweden, Finland and Iceland between 1856 and 1956 (Robertson, 2008). Leprosy was introduced into the Americas with European colonialists and the slave trade (Donoghue, 2019). Leprosy strains now found in the south eastern United States of America (USA) originated from Scandinavia, France and England (Donoghue, 2019) and some strains in South America are related to strains in West Africa (Schuenemann *et al.*, 2018) and ancient Europe (Benjak *et al.*, 2018). This illustrates that at least one ancient pathogen causing leprosy has been able to persist through the ages.

Today, Great Britain is used as an example of a country where leprosy was successfully eradicated in humans, with decades without autochthonous infections and low numbers of imported cases (Saunderson, 2008; Fulton *et al.*, 2016). The last autochthonous human leprosy case was reported in 1954 in Northwest England (Gill, Gill and Beeching, 2008). According to Fulton *et al.* imported cases do still occur in the country today with a large proportion of patients having been born in South Asia (Fulton *et al.*, 2016). Unfortunately, no details on the leprosy strains identified in these patients are available.

From the information readily available the human leprosy situation in several European countries such as Germany and France is very similar to that in Great Britain (Koch *et al.*, 2006; Hundeiker and Broemmelhaus, 2007; Ezzedine *et al.*, 2009; Reibel *et al.*, 2015; Gilsdorf and Robert Koch Institute, 2017). However, in addition to imported cases occasional autochthonous transmission of leprosy has been reported more recently in southern European countries such as Italy, Portugal, and Spain (Greco and Galanti, 1983; Massone *et al.*, 2012; Maritati and Contini, 2016; Ramos, Romero and Belinchón, 2016; Cusini *et al.*, 2017; Esgueva *et al.*, 2019). In Europe, no hosts for leprosy other than humans were described until 2014 (Meredith *et al.*, 2014; Schuenemann *et al.*, 2018).

1.1.2. *M. leprae* and *Mycobacterium lepromatosis*

Two mycobacterial species are causing leprosy, and can occur separately or as coinfections (Polycarpou, Walker and Lockwood, 2013). While *M. leprae* was identified in 1873 by Dr. Hansen (Rojas-Espinosa and Lovik, 2001), *M. lepromatosis* was only described in 2008 (Han *et al.*, 2008). The latter has not yet been isolated from ancient human samples and it is thus unknown for how long it has been causing disease in humans (Donoghue, 2019). Both leprosy bacilli primarily infect and grow in macrophages and Schwann cells (Duthie *et al.*, 2014).

M. leprae is a very slow growing bacterium, with a generation time of approximately 12- 14 days in animal models and tick cell lines (Blake *et al.*, 1987; Ferreira *et al.*, 2018). Freezing or heating above 37°C impairs *M. leprae* viability (<1% retained). This was determined by harvesting bacteria from mouse foot pads and storing them at varying temperatures before using the suspension to inoculate new mice and assess infection. The optimal growing temperature for *M. leprae in vivo* is thought to be 27-33°C, however no studies have been published that assessed the exact temperature at the site of growth within the host and related growth rates, as such data is extremely difficult to obtain (Truman and Krahenbuhl, 2001; Lastória and de Abreu, 2014b). Newly developed *in vitro* systems use this temperature recommendation and now hold the potential to set up experiments to determine the true optimal growth temperature (Ferreira *et al.*, 2018).

M. leprae has the smallest genome of all mycobacteria currently sequenced (3.3 Mb), and it contains almost as many pseudogenes as protein-coding genes (1300 and 1624, respectively). This reduced, highly conserved genome (less than 300 single-nucleotide polymorphisms (SNPs) even between distantly related strains) makes the bacterium dependant on a suitable host. *M. leprae* diverged from a common ancestor with *Mycobacterium tuberculosis* approximately 66 million years ago. Its adaption to an intracellular lifestyle is estimated to have occurred around 9 million years ago (Franco-Paredes and Rodriguez-Morales, 2016). Four SNP types (1-4) and 16 subtypes (A-P) have been identified today (Schuenemann *et al.*, 2018). Based on whole genome comparison *M. leprae* strains are also grouped into branches (0-5), which reflect greater complexity than the SNP types. These grouping systems have provided valuable insights into the worldwide geographical distribution and dissemination of the bacteria (Benjak *et al.*, 2018; Schuenemann *et al.*, 2018). Four branches are known to have been present in humans in Europe at some point in history (0, 2F, 3, 4). Branch 1 is only known to occur in South-East Asia, Eastern Africa, and South America, and branch 5 in some Pacific Islands (Schuenemann *et al.*, 2018).

It is thought that the rod shaped, acid-fast, microaerophilic, non-spore forming, non-motile, obligate intracellular bacilli have a low virulence and low transmissibility, and can thus persist in a host population almost unnoticed for long periods of time (Scollard *et al.*, 2006; Chinchilla, 2011). *M. leprae* is described as “well adapted, minimally toxic pathogen, capable of inhabiting various cells without marked injury or dysfunction” (Scollard, Truman and Ebenezer, 2015). *M. leprae* has an affinity for keratinocytes, macrophages and histiocytes is the skin and for Schwann cells of peripheral nerves. The bacilli interact with the host cell lipid metabolism to be able to survive (White and Franco-Paredes, 2015). In highly infected tissues the bacilli form clumps (globi) that can contain hundreds of bacilli (Reibel, Cambau and Aubry, 2015). *M. leprae* has a very thick cell wall containing components (lipoarabinomannan and phenolic glycolipid-I (PGL-I)) that can cause depression of

suppressor T-cells and inhibit macrophage bactericidal function. PGL-I induces the production of IgM antibodies in the host (Jurado *et al.*, 2015).

M. lepromatosis has been identified from human patients in Mexico, Brazil, the Caribbean, Myanmar, Singapore, Canada and India, and has been linked to diffuse lepromatous leprosy in particular (Han *et al.*, 2014; Ahuja *et al.*, 2018). No leprosy cases in sub-Saharan Africa have yet been linked to *M. lepromatosis* (Quao and Amankrah-Otabir, 2016). *M. lepromatosis* is very similar to *M. leprae* in all its main characteristics and can often only be differentiated by sequencing. It has been accepted as a separate species as there is an overall difference of about 9% between the whole genome sequences of both bacilli (Scollard, 2016). More differences exist between pseudogenes than between protein-coding genes. The two species diverged from a common ancestor about ten to fourteen million years ago (Han and Silva, 2014; Singh *et al.*, 2015). *M. lepromatosis* can heavily infect internal organs and is thus predicted to be able to grow at higher temperatures than *M. leprae*, to potentially be more virulent and to multiply more rapidly (Han *et al.*, 2008).

Until recently, growing either *M. leprae* or *M. lepromatosis in vitro* was simply impossible. This made both pathogens extremely difficult to study and is at least part of the reason why wide knowledge gaps still exist with respect to leprosy (Anand *et al.*, 2014; Reibel, Cambau and Aubry, 2015). Two animal models are established in which *M. leprae* can be propagated: the footpad of thymectomised mice and the nine-banded armadillo (*Dasypus novemcinctus*, NBA). However, both methods are cumbersome and time-intensive (Benjak *et al.*, 2018). In December 2018 *M. leprae* was successfully propagated the in *Ixodes scapularis* embryo-derived tick cell line IDE8. An increase in bacterial numbers occurred in these cells for at least 20 days (Ferreira *et al.*, 2018). This system holds the promise to make large scale *in vitro* propagation of leprosy bacilli and experimental manipulation finally possible.

1.1.3. Today's relevance of human leprosy

Leprosy is generally regarded as the "least infectious of all infectious diseases" (Rojas-Espinosa and Lovik, 2001). Humans are still seen as the main host and reservoir for leprosy bacilli (Reibel, Cambau and Aubry, 2015). Generally speaking, leprosy is a non-fatal disease (Bhat and Prakash, 2012). The majority of humans is thought to be genetically resistant to leprosy today and 90-95% of people will not develop clinical disease even when exposed to the pathogen (Scollard, Truman and Ebenezer, 2015; Quao and Amankrah-Otabir, 2016). The global World Health Organisation (WHO) human leprosy elimination target was met in 2000, with reported leprosy cases dropping to 1 per 10,000 (Fulton *et al.*, 2016). At the national level, most countries were able to reach this target by 2005 (WHO, 2017). Brazil still has not been able to meet this target (Salgado, Barreto, *et al.*, 2018). Lately, the WHO global strategy for leprosy control has moved on from the elimination of leprosy as a public health problem to aiming to reduce transmission and to achieve a reduction of individual disease

burden, particularly in terms of disability and discrimination (WHO, 2018a). Worldwide more than 200.000 new cases are still occurring each year. Annual statistics on leprosy occurrence are currently provided by 150 WHO member countries. The number of newly detected cases per annum and the number of leprosy patients under treatment at the end of the calendar year are reported (Table 1).

TABLE 1: HUMAN LEPROSY CASES REPORTED IN 2017 (WHO, 2018a)

WHO region	Number of cases under treatment at the end of 2017	Number of new cases detected during 2017
Africa	30,654	20,416
Americas	31,527	29,101
Eastern Mediterranean	4,405	3,550
South-East Asia	119,055	153,487
Western Pacific	7,040	4,084
Europe	32	33
Global total	192,713	210,671

Most cases (199,713; 94.8%) are reported from just 22 global priority countries, with India (126,164), Brazil (26,875), Indonesia (15,910), Bangladesh (3,754), the Democratic Republic of the Congo (3,649), and Nepal (3,215) reporting the highest case numbers (WHO, 2018a). One reason that has been proposed for regionally persisting human infections with leprosy are bad socioeconomic conditions (Schmitt *et al.*, 2010).

In Britain, similar to other European countries, up to approximately ten cases are notified each year, mainly in migrants or related to long term stay in high prevalence regions (Gill *et al.*, 2005). There is however some evidence that supports the possibility that leprosy can still occasionally be acquired locally in Western Europe (Greco and Galanti, 1983; Gill *et al.*, 2005; Ezzedine *et al.*, 2009; Maritati and Contini, 2016; Esgueva *et al.*, 2019).

The reporting system has come under criticism of potentially underrepresenting the true number of human leprosy cases worldwide (Smith *et al.*, 2015). Apart from the fact that only patients under treatment at the end of the calendar year are included, it has been pointed out that countries with well-structured health care systems, such as most European countries, the USA, Australia and Japan all report cases, while low-income countries did not report any, potentially not representing a true absence of disease but a lack of detection (Salgado, Barreto, *et al.*, 2018). Salgado *et al.* (2018) point out a need for active surveillance to identify all leprosy cases and provide them with adequate treatment, to reduce the risk of stagnating rates of new cases detected every year while exposure continues. Continued exposure to not identified cases also entails the risk that case numbers could rise again in the future (Smith *et al.*, 2015; Salgado, Barreto, *et al.*, 2018).

One big issue in controlling human leprosy is that even today the exact mechanisms of leprosy transmission, as well as the role of reservoirs, vectors and the environment are poorly understood (Smith *et al.*, 2015). Factors making the disease so very difficult to

understand include the long and variable incubation period, low incidence rates in exposed people, cases in people without known contact to active cases, and highly variable clinical presentations (Schmitt *et al.*, 2010; Smith *et al.*, 2015). The upper airway is believed to be the main site of entry into a new host, while both discharge from infected skin and airways are believed to be sources of viable bacteria for disease transmission (Silva *et al.*, 2013). Inhalation of aerosolized discharge is therefore currently seen as most likely route of transmission. Prolonged close contact to infected individuals may also be important, but currently proposed modes of transmission cannot explain the patchy distribution of leprosy, even when considering individual resistance (Franco-Paredes and Rodriguez-Morales, 2016).

Regarding leprosy resistance, the hypothesis has been put forward that a greater mortality in individuals co-infected with *M. leprae* and *M. tuberculosis* has driven the development of genetic resistance to leprosy in Europe (Donoghue, 2019). Effective Multi-drug (rifampicin, dapsone and clofazimine) treatment has been available worldwide since the 1980s, and is likely to have made a significant contribution to the reduction of case numbers. Human patients undergoing treatment are believed to no longer be a source of infection from day one of treatment (Lastória and de Abreu, 2014a). While no fully satisfactory vaccine has been produced yet, Bacille Calmette-Guerin (BCG) vaccination at birth can reduce the risk of leprosy by approximately 50%. It is already part of the vaccination policy in most leprosy-endemic countries and usually not recommended as control tool for leprosy specifically (Scollard *et al.*, 2006; WHO, 2017).

Continued efforts to control leprosy in humans are being made and are necessary, given the emergence of antibiotic resistant *M. leprae* strains (Benjak *et al.*, 2018), as well as the accepted status of leprosy as an emerging infectious disease in some countries such as the USA (Levis, Rendini and Martiniuk, 2018). Furthermore, ending social and legal discrimination and preventing lifelong disabilities in those affected by the disease are important current goals of human leprosy programs (WHO, 2018a). Leprosy continues to be an important human disease and great challenge to researchers and health managers.

Clinical symptoms in humans

Humans are a long-lived host species offering a range of different conditions to pathogens across their different organ systems. Individuals living for 115 to 122 years have been reported (Rafi and Alavi, 2017). The average global life expectancy at birth was 72 years in 2016 (WHO, 2018b). The average core body temperature of healthy individuals is given as 35 to 37.5°C (Geneva *et al.*, 2019). However, peripheral organs, such as the skin, are likely to have a lower temperature, influenced by the ambient temperature.

Most information on clinical leprosy in humans comes from patients infected with *M. leprae* (Han *et al.*, 2012). The incubation period for leprosy in humans is generally measured in

years (Blake *et al.*, 1987). The shortest incubation time for humans was described in a two-month-old child, but in most cases between six months and 20 years (mean incubation period two to four years) pass between initial infection and onset of clinical disease (Rojas-Espinosa and Lovik, 2001; Lastória and de Abreu, 2014b). Disease progression is slow (Ferreira *et al.*, 2018), but variable depending on the form of leprosy a patient exhibits and the occurrence of leprosy reactions. Information on exact timescales is rarely available, but the delay between onset of symptoms and diagnosis can be very long, i.e. 41.7 +/- 49.8 months (Li *et al.*, 2016), indicating that patients do not seek diagnosis when symptoms first occur or that initial misdiagnosis is possible. Pre-clinical identification of leprosy in humans is mainly achieved using serological methods (Anouk van Hooij *et al.*, 2016), but identification of infected individuals using molecular methods is also possible prior to the onset of clinical disease (Martinez *et al.*, 2014).

For a long time it has been argued that certain conditions (i.e. worm infestation, syphilis, influenza) which reduce the general resistance of an individual can correlate with the onset or intensification of leprosy lesions in human patients (Gordon-Napier, 1933; Sandre, Poenaru and Boggild, 2018). (Re-)activation of *M. leprae* infection in the postpartum period and during lactation, i.e. when the immunosuppressive status necessary to maintain pregnancy is reversed, has been described (Singh and Perfect, 2007). However, an infection with human immunodeficiency virus (HIV) was not linked to a higher rate of leprosy infection or clinical disease (Scollard *et al.*, 2006; Virmond, Grzybowski and Virmond, 2015). Antimicrobial and supportive treatment is usually initiated in patients as soon as the diagnosis is made and continued over six to 24 months (Reibel, Cambau and Aubry, 2015), thus the full natural course of infection is not usually observed and documented in humans in modern times.

When clinical leprosy develops its presentation is highly variable, shaped by the individual's genetically determined immune reaction towards the pathogen (Govindan *et al.*, 2018). The definitions for the main clinical forms of leprosy still used today were proposed by Ridley and Jopling in 1966 (Ridley and Jopling, 1966; Virmond, Grzybowski and Virmond, 2015). As the clinical presentation is so strongly linked to the host immune response, the five main categories to which human leprosy cases are assigned reflect a range from strong (tuberculoid, TT) to weak host immune response (lepromatous, LL), with three categories in between: borderline tuberculoid (BT), borderline borderline (BB), and borderline lepromatous (BL) (White and Franco-Paredes, 2015). Table 2 summarises the defining features of these main clinical forms of human leprosy.

TABLE 2: CLINICAL FEATURES OF THE FIVE MAIN FORMS OF HUMAN LEPROSY (Ridley and Jopling, 1966; Bhat and Prakash, 2012; Virmond, Grzybowski and Virmond, 2015; Gaschignard *et al.*, 2016)

Characteristic	TT	BT	BB	BL	LL
No. of lesions	One up to three	Up to 10	Ten to 30	More than 30, asymmetric	Innumerable, symmetrical
Lesion size	Large	Variable, some large	Variable	Small, some larger ones	Small
Lesion type	*Early:* Erythematous/ hypopigmented macule *Later:* Erythematous plaque	Macules or plaques, small satellite lesions possible	Irregular erythematous plaques, satellites, sometimes "hole-in-cheese" appearance	Macules, plaques, papules and nodules, not symmetric, some nodules dimpled in centre	Macules or papules, bilateral symmetric, erythematous
Lesion edge	Sharply raised, well defined	Slightly less clear cut than in TT	Vague or well-defined	Some definition	Vague
Lesion surface	Rough, pebbly, sometimes scaly	Less dry than TT, scaly	Dull or slightly shiny	Mildly shiny and succulent	Smooth, shiny
Peripheral nerves	Irregular thickening one or two nerves close to lesion	Multiple nerves close to lesion can be thickened	Nerve swelling less prominent	Nerve swelling from onset of lesions	Not thickened unless developed from BB/BL leprosy
Anaesthesia	Very marked	Marked	Moderate	Slight	Minimal
Hair growth	None	Markedly diminished	Moderately diminished	Slightly diminished	Not initially affected
Bacterial load in skin smear	None	None or 1+	1-3+	3-5+	6+, including globi
Immune response	T-helper cell (Th) 1 mediated, strong				Th 2 mediated, weak

At the TT end of the leprosy spectrum, lesions are usually restricted to the skin and peripheral nerves. Systemic leprosy in humans is mainly described on the LL end of the spectrum, i.e. in individuals with a weak cellular immune response. Then involvement of and bacterial presence in most internal organs is possible. In diffuse LL (DLL) AFB have been observed in skin, liver, spleen, lymph nodes, bone marrow, kidneys, adrenal glands and tracheal mucosa (Han *et al.*, 2008). Even in ancient remains of human lepromatous patients, high burdens of *M. leprae* are often found in the vomer (Mendum *et al.*, 2014). When LL leprosy affects the eyes, AFB are present in conjunctiva, cornea, ciliary body, ora serrata and sclera (Rathinam, Khazaei and Job, 2008).

Advanced, obvious clinical leprosy symptoms in humans are mainly the result of untreated LL (Ridley and Jopling, 1966; Han *et al.*, 2008). They include clawed hands due to lesions of the ulnar nerve, drop foot as a result of damage to the lateral peroneal nerve, madarosis (loss of eyebrows and eyelashes), and changes to the eye (corneal nerve beading, iris atrophy, iris pearls, iridocyclitis, keratitis) (Virmond, Grzybowski and Virmond, 2015). Bone changes can occur in hands and feet, in LL also in the rhinomaxillary area (Mariotti *et al.*, 2005). Ocular involvement is estimated to be present in 70-75% of human leprosy patients. Symptoms are severe in 10-50% of the patients and blindness occurs in about 5% (Grzybowski, Nita and Virmond, 2015). Leprosy is the leading cause of permanent disability due to an infectious disease (Fulton *et al.*, 2016).

Involvement of the peripheral nervous system is present in all forms of human leprosy (Soares *et al.*, 2017). Nerve damage can present as autonomic, sensory or motor disfunction, particularly sensory disfunction means that the host can suffer tissue damage without even realising it. Damage is caused either by an unrestricted replication of *M. leprae* or by the granulomatous host immune response to the presence of the pathogen (Duthie *et al.*, 2014). Very occasionally a purely neural form of leprosy without involvement of the skin is described (Primary neural leprosy, PNL) (Quao and Amankrah-Otabir, 2016).

Additional forms of leprosy that are described regularly are indeterminate leprosy (InL), histoid leprosy, and Lucio-Latapi leprosy. InL is a purely macular condition with hypopigmentation and slight anaesthesia. Macules can be ill-defined and bizarre with a smooth or scaly surface (Bhat and Prakash, 2012). Spontaneous resolution without treatment is described in this form. It can also develop into one of the five main forms (Rojas-Espinosa and Lovik, 2001; Quao and Amankrah-Otabir, 2016). Histoid leprosy is a sub-form of LL characterised by firm, reddish or skin coloured, dome shaped or oval papules or nodules, with regular contour, translucent shiny and stretched overlying skin (Massone, Belachew and Schettini, 2015; Talhari, Talhari and Penna, 2015). Lucio-Latapi leprosy is a polar type of LL. The skin shows a generalized infiltration without nodules, which give a brilliant and moist, healthy appearance. It was supposed to be unique to Mexico, but a few cases have been diagnosed in Brazil and India. (Virmond, Grzybowski and Virmond, 2015). Patients may develop fever, arthralgias, myalgias, and very painful red or purpuric macules of irregular shapes on lower legs, thighs, hip, trunk and upper limbs (Massone, Belachew and Schettini, 2015). The more recently described infection with *M. lepromatos*is mainly results in LL or DLL (Han *et al.*, 2012).

An additional complication of leprosy in humans are so called leprosy reactions. Two types are generally distinguished: reversal reactions (RR) and erythema nodosum leprosum (ENL). RR are caused by an immunocellular response of the host. They usually occur in TT or borderline patients. Clinical signs include swelling and reddening of pre-existing lesions. Necrosis and ulceration can occur in intense cases (Soares *et al.*, 2017). The reaction is

characterised by a delayed hypersensitivity to *M. leprae* antigen (Gell and Coombs type-IV reaction) and a sudden increase in the cell-mediated immune response (Fava *et al.*, 2012). ENL can occur when the cellular immune response to the presence of leprosy bacilli is weak, i.e. in LL cases. ENL are characterised by erythematous papules, nodules or plaques on lesions that can become necrotic and ulcerate, combined with systemic manifestations such as fever and inflammation of all bacteria containing organs (Soares *et al.*, 2017). Risk factors for developing such a reaction include high bacterial indices (3+), certain major hormonal changes in women (puberty, pregnancy, lactation), vaccinations, and emotional and psychological stress (White and Franco-Paredes, 2015; Sandre, Poenaru and Boggild, 2018). High levels of proinflammatory cytokines such as interferon-γ (INF-γ), TNF-α, IL-6, IL-12 and IL-1β are present in the serum of humans showing an ENL reaction (Bhat and Prakash, 2012; Fava *et al.*, 2012). During ENL reactions inflammation implying the presence of mycobacterial antigen can occur in a range of tissues, including peripheral nerves, joints, lymph nodes, oral cavity, larynx, liver, spleen, eyes, testes, kidney and bones (Soares *et al.*, 2017). In ocular ENL an influx of neutrophils on the background of macrophages packed with AFB has been described (Rathinam, Khazaei and Job, 2008).

Clinical differential diagnosis in humans

Clinical differential diagnosis for leprosy in humans are naturally manifold, given the diverse nature of the disease. Initially human leprosy can be mistaken for an allergic reaction, autoimmune disease, fungal infection, vitiligo, other mycobacterial infections, mucocutaneous leishmaniasis, syphilis or rheumatoid arthritis (White and Franco-Paredes, 2015). In the long run a range of non-infectious granulomatous dermatotic conditions, superficial non-granulomatous skin infections, non-infectious plaque and nodular diseases, cutaneous infectious granulomas and systemic disorders with diffuse cutaneous infiltration can look similar to different forms and stages of leprosy (Moschella and Garcia-Albea, 2016). Among the infectious diseases that could cause similar lesions are tuberculosis, atypical mycobacterial infection, leishmaniasis, syphilis, different forms of blastomycosis and African histoplasmosis (Moschella and Garcia-Albea, 2016).

Histological presentation of leprosy in humans

Histological descriptions in humans are often focussed on the skin, but according to Ridley and Jopling (1966) lesions in other tissues are essentially similar. Leprous granulomas are rarely found in the superficial dermis only, usually dermis and sometimes subcutis are involved. The presence of AFB inside a nerve is diagnostic of leprosy (Massone, Belachew and Schettini, 2015).

The clinical classification of leprosy lesions does not always correlate with the histological features. Ridley-Jopling categories assigned to a patient clinically and histologically differ in 30% to 62% of cases. Discrepancies mainly occur in borderline cases (Massone, Belachew and Schettini, 2015). Nevertheless, the five main histological categories of leprosy lesions

have the same names as the clinical categories. Criteria correlated with the histological progress and classification of leprosy lesions are: “foam cells, large globi, epithelioid cells, Langhans giant cells, lymphocytes, plasma cells, fibroblasts, a clear subepidermal zone, the cellular cuffing of nerves, and infiltration of nerves” (Ridley and Jopling, 1966). Table 3 details the characteristics of the different histological leprosy presentations. Histological features may be in between Ridley Jopling categories. This can be described by combining the categories, i.e. TT-BT (Massone, Belachew and Schettini, 2015).

TABLE 3: HISTOLOGICAL FEATURES OF THE FIVE MAIN FORMS OF HUMAN LEPROSY (Ridley and Jopling, 1966; Bhat and Prakash, 2012; Massone, Belachew and Schettini, 2015)

Characteristic	TT	BT	BB	BL	LL
Extent of infiltrate	Dermis to epidermis	Dermis, seldom reaching epidermis	Spares epidermis, but epidermis can be atrophic	Spares epidermis, formation of a small band of collagen (Unna band)	Clearly separated from epidermis (Unna band), can reach deep into dermis
Cells observed	Epithelioid cells, Langhans giant cells, dense lymphocyte infiltration	Epithelioid cells, Langhans giant cells, dense lymphocyte infiltration	Epithelioid cells, some lymphocytes diffusely spread	Histiocytic cells, no foamy cells and scanty lymphocytes OR histiocytes with foamy changes, dense areas of lymphocyte infiltrate	Histiocytes with fatty changes, foam cells, multinucleate globe, few lymphocytes
Nerve involvement	Nerve bundles unrecognisable within granuloma	Swollen, infiltrated	Moderate Schwann cell proliferation	Perineural lymphocyte cuffs, loss of nerve bundle structure	Some structural damage, no infiltration
BIn	0	0-2 granuloma, 1-3 nerve bundles	3-4	5	5-6
PCR from lesion	Often negative	Positive in 50% of cases	Positive in most cases	Almost always positive	Always positive
Dominant Th type	CD4+				CD8+

The bacterial index (BIn) was introduced to comparably quantify AFB seen in skin smear samples from human leprosy patients (Virmond, Grzybowski and Virmond, 2015) or in histopathologic sections of granulomas (Massone, Belachew and Schettini, 2015). It is

expressed on a logarithmic scale (Ridley and Jopling, 1966; Talhari, Talhari and Penna, 2015):

- 1= at least 1 bacillus in every 100 fields
- 2= at least 1 bacillus in every 10 fields
- 3= at least 1 bacillus in every field
- 4= at least 10 bacilli in every field
- 5= at least 100 bacilli in every field
- 6= at least 1000 bacilli in every field.

InL is histologically often indistinguishable from other forms of chronic dermatitis. Lymphocytes and histiocytes are localised around skin annexe. Fibrocytes are often increased and perineural cuffing or increased cellularity in a nerve bundle can be present. AFB are scarce or absent, a polymerase chain reaction (PCR) test for DNA from lesion tissue is only positive in a small number of cases (Massone, Belachew and Schettini, 2015). Histoid leprosy presents with spindle-shaped cells, which gave this sub form its name. Small numbers of foamy macrophages and unusually large numbers of AFB can be present (Massone, Belachew and Schettini, 2015; Talhari, Talhari and Penna, 2015). Lucio-Latapi leprosy presents with epidermal necrosis, ulceration, features of DLL, including numerous AFB, and a panvasculitis of superficial and deep vessel (Massone, Belachew and Schettini, 2015).

RR are histologically characterised by poorly delimited granulomas associated with intracellular and interstitial oedema, fibrin deposition, necrosis and varying degrees of epithelial hyperplasia (Soares *et al.*, 2017).The number of lymphocytes in the dermis is increased, and Langhans giant cells may be observed (Fava *et al.*, 2012). ENL presents histologically as an acute or subacute non-granulomatous inflammatory reaction with vascular proliferation, endothelial swelling, disorganisation of pre-existing granulomas and formation of micro-abscesses (Soares *et al.*, 2017).

Histological differential diagnosis for leprosy include, on the TT-BT end of the spectrum sarcoidosis, tuberculosis, leishmaniasis, and secondary syphilis (Massone, Belachew and Schettini, 2015). On the BL-LL end of the spectrum xanthomas and xanthogranulomas, post–kala-azar dermal leishmaniasis, paraffinoma, and rarely infections caused by other nontuberculous (atypical) mycobacteria can look similar (Massone, Belachew and Schettini, 2015)

Diagnosing leprosy in humans

Leprosy in human patients is usually diagnosed clinically, once symptoms of the disease become obvious. (Lumpkin III *et al.*, 1983; Rojas-Espinosa and Lovik, 2001; WHO, 2016a). In non-endemic areas the lack of awareness of clinicians regarding the symptoms of leprosy is an important cause for delayed or non-diagnosis (Moschella and Garcia-Albea, 2016).

In most cases (90%) dermatological signs are the main indicators for leprosy, while about 10% of patients present with neurological signs only (Reibel, Cambau and Aubry, 2015). Hypopigmented or reddened anaesthetic skin lesions, thickening of peripheral nerves and the presence of AFB are the cardinal symptoms of leprosy in humans (ILA Technical Forum, 2002; Quao and Amankrah-Otabir, 2016). For field diagnosis and treatment purposes human leprosy patients are usually separated into multibacillary (MB) and paucibacillary (PB) cases, based on the number of observed skin lesions (<5 = PB, >5= MB) (Quao and Amankrah-Otabir, 2016; WHO, 2016a). However, some local adaptions in determining whether a patient has a MB or PB form of leprosy exist, where, for example, the number of affected body areas is assessed rather than the number of lesions alone (Rao *et al.*, 2005). Additional grading systems exist to class the disabilities a leprosy patient has developed (van Brakel, Reed and Reed, 1999). The more detailed histological Ridley-Jopling classification system is mainly used for research purposes (Ridley and Jopling, 1966; Reibel, Cambau and Aubry, 2015).

Available complementary techniques for the diagnosis of leprosy include skin smear and fine needle aspirate microscopy, histopathology, PCR, serology, Mitsuda intradermal reaction, immunohistochemistry, imaging, and electromyography (Scollard *et al.*, 2006; White and Franco-Paredes, 2015; Baddam *et al.*, 2018). Availability of all tests depends strongly on local infrastructure and expertise.

The RLEP qPCR is the best currently available molecular method to confirm a leprosy diagnosis, when *M. leprae* is the causative agent (Braet *et al.*, 2018). Highly specific PCR protocols to identify *M. lepromatosis* DNA are also established (Han *et al.*, 2008; Vera-Cabrera *et al.*, 2015). Molecular methods are however usually limited to well-equipped laboratories. They can be performed using skin biopsies, slit skin smear and fine-needle aspirates from active lesions. Specificity is 100%, and in MB cases sensitivity is equally high. In PB cases however, sensitivity is lower (84.6%), and false negative results will occur (Baddam *et al.*, 2018; Braet *et al.*, 2018). A recent review, not taking different forms of leprosy into consideration summarised the diagnostic sensitivity of conventional PCR techniques in humans to be 75.3% (95% CI 67.9 to 81.5%, included studies reporting 50% to 93% sensitivity) and the specificity as 94.5% (95% CI 91.4 to 96.5, included studies reporting 90% to 100%) (Gurung *et al.*, 2019). Whole blood is another sample type to which PCR methods have been successfully applied to confirm a leprosy diagnosis. In one study *M. leprae* DNA was successfully isolated from blood samples of 47 out of 49 (95.92%) MB patients and from 70% (21/30) of the included PB patients (Wen *et al.*, 2013). Molecular methods have also been successfully applied to other sample types such as nasal swabs and sputum. These sample types are of slightly lower diagnostic value, as it is difficult to discern a transient presence of leprosy bacilli following contact from a true infection in which the bacteria have broken through the host defence (Wen *et al.*, 2013). Molecular methods,

first established in 1989, are mainly used in surveillance efforts driven by well-funded research groups and not widely part of the diagnostic toolkit available to health workers in the field (Scollard *et al.*, 2006; Fontes *et al.*, 2018)

Huge efforts have been made to develop serological tools suitable not only to confirm the clinical diagnosis of leprosy but also to identify infected and exposed individuals before the onset of clinical disease as part of surveillance efforts. These efforts aim to enable an early delivery of treatment and to break transmission chains (Lastória and de Abreu, 2014b; Corstjens *et al.*, 2019). The longest and most widely used parameter is leprosy specific anti-PGL-I antibody (αPGL-I) titres (Spencer and Brennan, 2011). Positive αPGL-1 titres alone do not imply that a patient will develop clinical leprosy (Wen *et al.*, 2013; van Hooij *et al.*, 2018). Alone PGL-I is mainly useful to identify or confirm MB cases (Bobosha *et al.*, 2014). Even in these cases the sensitivity differs between study populations and ranges from 75 to 100% (Lastória and de Abreu, 2014a; van Hooij *et al.*, 2018). Other proteins and antigens used in leprosy diagnostics include for example leprosy Infectious Disease Research Institute (IDRI) diagnostic-1 (LID-1), protein advances for the diagnosis of leprosy (PADL) and antigen 85B (de Souza *et al.*, 2014; Duthie *et al.*, 2014; de Santana *et al.*, 2018). Most recently developed tests combine the detection of αPGL-I IgM (humoral immune response) and IFN-c induced protein (IP-10), and C reactive protein (CRP) (cellular immune response) to improve the ability of serological tests to identify PB cases (van Hooij *et al.*, 2018; Corstjens *et al.*, 2019). These additional parameters can increase the sensitivity for the detection/confirmation of PB cases to 63-80%, depending on assessed population (van Hooij *et al.*, 2018). Lateral flow assays using up-converting phosphor reporter technology (UCP-LFA) have enabled field friendly, relatively cheap, quantitative serological testing (Bobosha *et al.*, 2014). They offer a diagnostic aid that does not require specialist knowledge and can be made available even in regions where other tools, like histopathology or molecular assays cannot be accessed (Corstjens *et al.*, 2019). As the sensitivity and specificity of serological tests continues to be improved, their value and relevance, particularly for large scale population screening on minimally invasive samples (fingerstick blood), is likely to continue to increase (Corstjens *et al.*, 2019).

Histopathological assessment of Ziehl-Neelsen (ZN) or Fite-Faro (FF) stained slit skin smear, fine needle aspirates, or biopsies taken from lesions, allows experienced investigators to establish the presence and number of AFB and some frequently present cell types and thus assign a category along the histological Ridley and Jopling spectrum (Ridley and Jopling, 1966; Scollard *et al.*, 2006). However, PB cases are easily overlooked (Massone, Belachew and Schettini, 2015) and in the absence of lesions deciding on a site for a biopsy or getting consent to take such is likely to be difficult. Histopathology, while important in clinical cases, therefore plays no or a very limited role in active surveillance efforts. Immunohistopathological techniques can increase the sensitivity and specificity of the

histopathological diagnostics, but they require specialist knowledge and adequate laboratory facilities (ILA Technical Forum, 2002).

The Mitsuda intradermal reaction is not diagnostic of leprosy or exposure to leprosy bacilli, but assists in placing a patient within the leprosy spectrum (Scollard *et al.*, 2006). Another simple test occasionally described is the reduced reaction of leprous hypochromic lesions to the application of histamine due to the destruction of local sympathetic nerve fibres (Talhari, Talhari and Penna, 2015). Imaging technologies are mainly employed to assess the effects the disease has already had on a patient following the confirmed diagnosis of leprosy (White and Franco-Paredes, 2015).

1.1.4. Leprosy as a multi host disease

Many animal pathogens can infect and cause disease in multiple hosts. Factors influencing which potentially susceptible hosts a pathogen, or specific strains of a pathogen, will affect and how it will affect them, depend on host and pathogen range, inherited traits, life history, and external requirements for transmission being met (Bowden and Drake, 2013).

In the 1970s it was realised that humans were not the only natural host for *M. leprae*, during attempts made to establish an animal model for human leprosy (Scollard et al. 2006). Natural infection in a non-human animal with *M. leprae* was first described in nine-banded armadillos (*Dasypus novemcinctus,* NBA) in Louisiana, USA (Walsh, Meyers and Binford, 1986) and has since been confirmed for a range of species (see below).

M. leprae strains isolated from species other than humans belong to branches 3I, 4 and 0. The same strains have been or are present in the human populations in each given geographic area (Schuenemann *et al.*, 2018). Branch 3I has been isolated in NBA and ERS, while branch 4 has been isolated from a chimpanzee (Sierra Leone) and a soothy mangabey monkey (*Cercocebus atys*, West Africa), and branch 0 from a cynomolgus macaque (*Macaca fascicularis*) from The Philippines (Schuenemann et al., 2018).

Leprosy in armadillos

NBA are THE armadillo of leprosy research, both due to their use in experimental settings and because natural infection has been described most frequently in this species (Balamayooran *et al.*, 2015). They have a shorter lifespan than humans and their core body temperature is lower than that of man with 30 to 35°C (McDonald and Larson, 2011). Mortality rates in juvenile NBA are relatively high, compared to those observed in adults, as is the case for many wildlife species. NBAs that reach maturity have been reported to live for seven to 20 years in the wild. The oldest reported NBA in captivity lived to an age of 23 years (McDonald and Larson, 2011).

Other armadillo species have been found to be susceptible to experimental *M. leprae* infection or harbour *M. leprae* DNA as well. These include Northern long-nosed armadillos

(*Dasypus sabanicola*), Southern long-nosed armadillos (*Dasypus hybridus*) and six-banded armadillos (*Euphratus sexcinctus*) (Storrs, 1978; Frota *et al.*, 2012). Efforts to identify *M. leprae* in small numbers of wild Southern naked-tailed armadillos (*Cabassous unicinctus*) and Greater naked-tailed armadillos (*Cabassous tatouay*) were unsuccessful (Pedrini *et al.*, 2010), but this does not exclude that individuals from even more of the 21 extant armadillo species (Gardner, 2006) could be susceptible to an infection with *M. leprae* under certain circumstances.

It is believed that armadillos, who are only found in the Americas, first acquired *M. leprae* from a human source (European explorers, slave trade) and that the infection is now sustained in the armadillo populations (Cardona-Castro *et al.*, 2009; Han and Silva, 2014). This is for example supported by the fact that in Louisiana human leprosy was reported as early as 1766 and the first leprosarium was opened in 1785. Positive serum samples from wild armadillos have tested positive as far back as 1961, though sample availability is likely to be a limitation here (Blake *et al.*, 1987). The low diversity in *M. leprae* strains identified in wild NBA in the Southern USA (3I-2-v1, 3I-2-v14, 3I-2-v13, 3I-2-v15) is thought to imply that interspecies transfers from humans to armadillos have been limited to rare, uncommon events, and that highly efficient intra-species transmission has led to leprosy distribution pattern seen in NBA today (Truman *et al.*, 2011; Sharma *et al.*, 2015). SNP type 3 *M. leprae* strains have also been identified in a small number of NBA in Brazil (Frota *et al.*, 2012). *M. leprae* strain diversity in humans in the Americas is much higher than in NBA (Sharma *et al.*, 2015; Schuenemann *et al.*, 2018).

In wild NBAs in Louisiana and Texas the prevalence for αPGL-I was determined to be 16% in the overall population and 28.5-32% if only adult armadillos were considered (Truman *et al.*, 1991). Histopathological prevalence in the Louisiana population had been determined to be 4% some years earlier (Walsh, Meyers and Binford, 1986). Other authors reported a seroprevalence of αPGL-I of 19% and a histopathological prevalence of 3% in the Louisiana armadillo population (no age correction) (Paige, Scholl and Truman, 2002). In a longitudinal study from Mississippi annual seroprevalence in the NBA population ranged from 4.5-15% but unfortunately the serological status varied in many animals and none were seropositive for more than three consecutive years (Williams and Loughry, 2012). In Brazil, *M. leprae* DNA was isolated from 21% of the animals in a mixed sample of six- and nine-banded armadillos, with the ear tissues having the highest rates of detection compared to liver, nose and spleen biopsies (Frota *et al.*, 2012). The disease seems to have minimal impact on the individual in wild NBA (Morgan and Loughry, 2009). Paige *et al.* (2002) estimated a serologic and histopathological incidence density to range from 0.47 to 3.5 per 1,000 animal-days, and therefore relatively high inter-armadillo transmission rates, with direct contact and habitat sharing being the most likely routes of infection (Paige, Scholl and Truman, 2002). Alternatively, vector born, environmental or food (insects) based transmission route have

been proposed (Walsh, Meyers and Binford, 1986). Under laboratory conditions NBA can be infected with *M. leprae* via intravenous, intradermal, percutaneous and respiratory administration of the bacteria (Pena, Sharma and Truman, 2018). *M. leprae* can be isolated from all organs in infected NBA, but cooler body regions, like ears, nose, tongue, footpads, lungs and bronchi show higher numbers of bacteria (Pena, Sharma and Truman, 2018).

NBAs always give birth to identical quadruplets. While individuals from the same litter, when infected with *M. leprae* will all show similar numbers of bacteria over time, there are differences between litters (Pena, Sharma and Truman, 2018). These can be grouped in three categories: High responders (high bacterial count), low responders (low bacterial count), and resistant individuals (about 15-20% of all individuals) (Truman *et al.*, 2014; Pena, Sharma and Truman, 2018). Some of these resistant armadillos have SNPs in toll-like receptors in similar locations as resistant humans (Pena, Sharma and Truman, 2018).

Clinical signs and histological presentation in NBA

The initial discovery of leprosy in NBA followed observations of enlarged inguinal lymph nodes in wild caught animals. The lymph nodes contained large numbers of AFB (Walsh, Meyers and Binford, 1986).

Naturally acquired leprosy infection is rarely described in juveniles or subadult NBAs, and most are thought to be older than two years at the time of initial disease detection, with the incubation period estimated to be 12-24 months (Walsh, Meyers and Binford, 1986; Oli *et al.*, 2017). Leprosy appears to be a disease of older NBA (Morgan and Loughry, 2009). There is little evidence of clinical disease, deformities or impairment due to leprosy in wild NBA and endemic infection does not appear to put a population at a disadvantage (Truman, 2005; Morgan and Loughry, 2009). Often NBA infected with *M. leprae* cannot clinically be distinguished from uninfected animals (Truman, 2005). In a population with a seroprevalence of 20% only 5% showed clinical signs of disease, and other studies also showed 10% or less of infected NBAs to be clinically diseased (Truman *et al.*, 1986; Walsh, Meyers and Binford, 1986). If wild NBA do develop clinical signs of leprosy they are usually few (Cardona-Castro *et al.*, 2009), and may be nodule-like lesions or non-specific abrasions around eyes, nose and feet (Frota *et al.*, 2012; Sharma *et al.*, 2013). Some studies have found more females to be infected than males, in others the sex ratio was balanced (Morgan and Loughry, 2009).

In experimentally infected (intravenous administration of a bacillary suspension containing $2x10^9$ viable bacilli), wild caught, adult NBA abnormalities in nerve conduction velocity can be observed within 12 months post infection, and disseminated disease is present 18-24 months post infection (Sharma *et al.*, 2018). The earliest neurological effects have been documented in an individual 90 days post infection and were correlated with a detectable immune response (Scollard, Truman and Ebenezer, 2015). αPGL-I can be detected earlier than clinical signs of disease or bacterial DNA in NBA, usually within a third of the time

required for the development of clinical signs (Truman *et al.*, 1991; Loughry *et al.*, 2009). Deaths from leprosy or its complications have been reported in experimentally infected NBA 15 to 41 months post infection (average survival time 31 months, n= 8). However, deaths can occur in NBA under laboratory conditions in a similar timeframe, even if infection with leprosy bacilli is unsuccessful (n= 5; Storrs *et al.*, 1974). NBA can show the full spectrum of immunological responses described in humans when infected with *M. leprae* (TT to LL), though the majority (~70%) of armadillo cases appear to be MB, and at the BL-LL end of the spectrum (Truman, 2005; Sharma *et al.*, 2013). Increased metabolic rates, asymmetrically distributed, focal, ulcerative dermatitis, extensive neurological involvement, anaemia and compromised liver and renal function are reported for NBAs under experimental conditions (Morgan and Loughry, 2009; Sharma *et al.*, 2013; Truman *et al.*, 2014).

Differential diagnosis for leprosy in NBA is not currently discussed in the literature and will depend on the individual disease presentation observed in a specific NBA.

As with clinical lesions, all five main types of histological lesions known in humans have been described in armadillos, with MB presentation being more frequent (Truman, 2005; Sharma *et al.*, 2013). Histological changes described in naturally infected armadillos include lesions containing macrophages filled with AFB in the skin, here often arranged in globi, as well as AFB in small and large peripheral nerves, lymph nodes, histiocytes in spleen and liver, and pulmonary macrophages (Walsh, Meyers and Binford, 1986; Rojas-Espinosa and Lovik, 2001). AFB in dermal nerves were only found in the latest stages of natural infection, long after the NBA had seroconverted (Paige, Scholl and Truman, 2002).

Leprosy bacilli can be found in all organs in armadillos, if the infection can progresses for long enough, but cooler body regions tend to exhibit greater involvement (Truman, 2005). Internal tissues of the nose are frequently affected by leprosy, but apparently are not the primary focus of infection. The tongue is also regularly involved, but lesions appear to be transient (Walsh, Meyers and Binford, 1986). Lepromatous placentitis and intrauterine foetal infections are possible in armadillos, and AFB may be found in milk and mammary glands (Walsh, Meyers and Binford, 1986; Rastogi, Legrand and Sola, 2001). Ocular involvement has also been described, with AFB in macrophages in all ocular tissues but for lens, retina, aqueous and vitreous humours (Hobbs *et al.*, 1978). When looking at six leprosy infected armadillos, Frota *et al.* found *M. leprae* DNA in the ear tissue of all six, in the nose and liver of five and in the spleen of three, implying that distribution through the body is uneven, even in systemic disease (Frota *et al.*, 2012).

Diagnosing leprosy in NBA

Serological tests, aiming to identify αPGL-I were trialled and used in NBAs early on (Truman, 1985; Truman *et al.*, 1986), along with histopathological confirmation of the presence of AFB in a variety of tissues (Walsh, Meyers and Binford, 1986; Stallknecht *et al.*, 1987). It has

been shown that αPGL-I begin to rise before bacteria can be detected in skin scrapings or ear biopsies in this species. PGL-I IgM increase with increasing bacterial loads and persists throughout the infection (Truman, 2005). While serology is the most sensitive diagnostic tool in NBA, surveillance efforts attempting to identify wild armadillos infected with leprosy have also used PCR methods to identify individuals carrying the bacteria (Sharma *et al.*, 2015). Especially where opportunistic sampling is used, it might be more feasible to acquire tissue samples for PCR and (immuno-)histopathological assessment than to collect serum (Pedrini *et al.*, 2010; daSilva *et al.*, 2018). In one study an 85B qPCR (single copy *M. leprae* gene), an RLEP qPCR (multi-copy *M. leprae* gene) and a combination of both was used to identify *M. leprae* DNA in buccal swabs of experimentally infected NBA. This sample type was however not suitable to identify all infected NBA (85B: sensitivity 0.36, specificity 1.00; RLEP sensitivity 0.72, specificity 0.60; 85B+RLEP sensitivity 0.60, specificity 0.85) with 28-64% of NBA testing false negative (Housman *et al.*, 2015). Other armadillo species harbouring leprosy bacilli have been identified using molecular methods. *M. leprae* DNA was isolated from a range of tissues (ear, nose, liver, spleen) in these (Frota *et al.*, 2012).

Leprosy in squirrels

Naturally acquired infection with *M. lepromatosis* (Meredith *et al.*, 2014; Simpson *et al.*, 2015) and *M. leprae* (Avanzi *et al.*, 2016) has recently been described in wild ERS in the British Isles as part of ongoing disease surveillance efforts. Naturally occurring leprosy has not been described in any other squirrel species. Targeted screening efforts were limited at the start of this study. Four grey squirrels were screened for leprosy with the same methods that revealed disease presence in ERS, and neither mycobacterial species was detected (Avanzi *et al.*, 2016). However, hibernating Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) and Siberian chipmunks (*Tamias sibiricus*) were successfully experimentally infected with AFB isolated from human leprosy patients in the 1970s/80s (Lew, Yang and Pyun, 1974; Galetti, Cavicchi and Ussia, 1982). In Thirteen-lined ground squirrels bacteria multiplied in all organs but counts were higher in the skin than in visceral organs (Galetti, Cavicchi and Ussia, 1982). However, these species have not been further used as leprosy models, and no coordinated screenings of wild populations have been published.

The *M. leprae* strain isolated from ERS is closely related to ancient human strains from England (Great Chesterford, Winchester) and Denmark (Odense), and current strains isolated from human patients in Brazil (Avanzi *et al.*, 2016; Benjak *et al.*, 2018; Schuenemann *et al.*, 2018). Only a single strain of *M. leprae* belonging to branch 3I could be sequenced from ERS so far (Avanzi *et al.*, 2016). Beyond its relatedness to human strains it is also of the same sequence type as strains isolated from NBA (Avanzi *et al.*, 2016). It is hypothesised that ERS first became infected with leprosy from a human source (Avanzi *et al.*, 2016; Schuenemann *et al.*, 2018). Current evidence is too scarce to determine when

ERS first became infected with *M. leprae* and no evidence is available for the presence of leprosy in ERS in Scandinavia or the Baltic region, two areas that have been implicated as potential points of origin for *M. leprae* strains present in Britain (Inskip *et al.*, 2017).

Unfortunately, no similar ancient DNA data exists yet to clarify the origin of *M. lepromatosis* strains found in ERS. It is however likely, based on comparisons made between strains in British and Irish ERS, that *M. lepromatosis* was present in ERS before they were reintroduced into Ireland 200 years ago. Infected ERS could have brought the bacteria with them during the reintroduction (Avanzi *et al.*, 2016). Locally acquired human infection with *M. lepromatosis* has not been described in the UK to date. The strains present in ERS in the UK and in human patients in Mexico are thought to have diverged from their most recent common ancestor about 27,000 years ago (Avanzi *et al.*, 2016).

SNPs identified in the toll-like receptor I (TLR-I) of ERS that seem to correlate with disease resistance are different from those identified in humans and armadillos (Avanzi *et al.*, 2016). ERS can develop clinical disease when infected with leprosy bacilli, but bacteria have also been isolated from apparently healthy ERS (Avanzi *et al.*, 2016).

Clinical signs in ERS

The most prominent clinical signs of leprosy described in ERS are bilateral areas of alopecia and cutaneous swelling on snout, lips, eyelids, ears and distal aspects of the limbs (Meredith *et al.*, 2014). Clinical signs are highly similar between ERS infected by *M. leprae* and those infected by *M. lepromatosis* (Avanzi *et al.*, 2016). Simpson *et al.* (2015) described a crusty thickening of the ears with some keratinisation and wart-like protuberances in two ERS from the Isle of Wight infected with *M. lepromatosis*. This implies, that more variations in clinical presentation of ERS may exist. Skin conditions in ERS that are relevant differential diagnosis for leprosy, are summarised in Table 4 (p. 21).

Histological presentation in ERS

The first leprosy lesions in Scottish ERS, caused by *M. lepromatosis* were described as presenting with large numbers of AFB and consistent with DLL (n=3) (Meredith *et al.*, 2014). Leprosy lesions caused by *M. lepromatosis* in ERS on the Isle of Wight were also described as consistent with DLL, despite the unusual clinical presentation and only moderate numbers of AFB (n=3) (Simpson *et al.*, 2015). Additional *M. lepromatosis* cases (tissue= pinna) from Scotland with typical clinical lesions (n=4), were described as LL (75%) or BL (25%) and on the Isle of Wight (n=1, again with atypical clinical presentation) as LL but with only sparse numbers of AFB in bands of macrophages close to the cartilage of the pinna and in the spleen. In an Irish sample of two *M. lepromatosis* positive ERS without clinical signs, only skin from the front foot (dorsum and footpad) were available. In only one of these ERS were AFB observed and, based on the description provided, the lesion appeared to be indeterminate (Avanzi *et al.*, 2016).

TABLE 4: CONDITIONS CAUSING SKIN LESIONS IN ERS

Diagnosis (causative agent)	Clinical lesion description	Histological lesion description	Source
Atypical histiocytosis (unknown)	Cutaneous, subcutaneous and/or internal swellings and nodules	Sheets of atypical round cells and multinucleated giant cells	(Smith *et al.*, 2017)
Malignant melanoma (non-infectious)	Masses on eyelids	Highly pleomorphic epithelioid cells, some with melanin granules	(Fukui *et al.*, 2002)
Poxvirus infection (Squirrelpox virus, SQPV)	Erythematous scabs on periorbital skin and feet, skin ulceration	Mixed inflammatory cell dermatitis, ulceration, ballooning degeneration of epidermal cells	(McInnes *et al.*, 2009)
Poxvirus infection (Berlin squirrelpox virus)	Exudative and erosive dermatitis with serocellular crusts at auricles, noses and digits, tail and perineum	Ballooning degeneration of epidermal keratinocytes, intracytoplasmatic inclusion bodies, epidermal ulceration, secondary bacterial infection	(Wibbelt *et al.*, 2017)
Fatal exudative dermatitis (*Staphylococcus aureus*)	Exudative scabby lesions around mouth, nose and eyelids, inflammation and sloughing of foot skin	Exudative, ulcerative, necrotic dermatitis with epidermal hyperplasia and hyperkeratosis	(Simpson *et al.*, 2013; Blackett *et al.*, 2018)
Abscess (for example *Yersinia enterocolitica*)	Facial abscesses	Dermal necrosis	(Simpson *et al.*, 2013)

Twenty-five squirrels from which *M. leprae* DNA had been isolated were assessed histologically prior to this study, eight of which had clinical lesions (Avanzi *et al.*, 2016). Out of the 17 clinically negative, PCR positive ERS only six showed histological features indicating leprosy in the form of a mild chronic perineuritis in the pinna or muzzle. In the other 11 no histological features indicating leprosy were found. The clinically diseased animals were again classed as either LL (n=6, BIn 2+/3+) or BL (n=2, BIn 1+/2+), and neuritis was noted in all these cases at least in the most affected sections. The study also indicated that the anatomical location of the skin sample was influencing the intensity of AFB and pathological changes observed (Avanzi *et al.*, 2016). Ulcerations, but not vasculitis, have been described in squirrels with clinical leprosy lesions infected with either *M. lepromatosis* or *M. leprae* (Avanzi *et al.*, 2016).

Diagnosing leprosy in ERS

Leprosy in ERS was initially diagnosed histologically in animals presenting with unusual clinical skin lesions at post mortem examination. Subsequent PCR analysis and sequencing of the product confirmed the presence of *M. lepromatosis* (Meredith *et al.*, 2014). The same methods were used consecutively on the Isle of Wight to identify the disease (Simpson *et al.*, 2015).

The largest currently published study on leprosy detection in opportunistic samples from dead squirrels combined histological and PCR analysis with sequencing of the isolated bacterial DNA and serological screening of body cavity fluid collected post mortem for *M. leprae*- and *M. lepromatosis*-specific αPGL (Avanzi *et al.*, 2016). The latter was less effective in identifying cases than histology and PCR. An ML Flow test developed by KIT (Royal Tropical Institute) Biomedical Research in Amsterdam developed for diagnosing leprosy in humans had been used. Later post mortem screening efforts for leprosy in squirrels have relied primarily on PCR testing (Butler *et al.*, 2017; Schilling, Avanzi, *et al.*, 2019).

Leprosy in other species

Clinical leprosy has not been reported among free-ranging primates. However, individuals of some species can be infected experimentally and in a few instances wild caught non-human primates have developed clinical leprosy in captivity, without being experimentally infected (Truman and Fine, 2010).

Naturally acquired leprosy infection was first diagnosed in a captive sooty mangabey monkey in 1979. A contact animal developed disease seven years later. Leprosy has also been described in a white-handed gibbon (*Hylobates lar*) in Malaysia, that was kept at a leprosarium. The animal was experimentally inoculated with leprosy bacilli, but did not develop leprosy until 15 years after the experiments. In the meantime, it had been tended by human leprosy patients. It can therefore not be said whether the infection was experimental or natural (Meyers *et al.*, 1991).

Clinical leprosy was also observed in a wild caught chimpanzee (*Pan troglodytes scheinfurthii*) kept in a research facility. A West African *M. leprae* strain (Branch 4) was isolated, implying an infection acquired around the time of capture and an incubation period of about 30 years. The animal responded well to multi drug treatment as used for humans. It cannot be discerned whether the chimpanzee was infected by a strain circulating in the primate population or from a human source during initial captivity (Suzuki *et al.*, 2010).

Natural infection in a cynomolgus macaque in the Philippines was described in 1998 (Valverde *et al.*, 1998). The *M. leprae* strain isolated from the cynomolgus macaque from The Philippines is most closely related to a human *M. leprae* strain belonging to branch 0 from New Caledonia, while strains isolated from a different chimpanzee than those mentioned above and a sooty mangabey monkey belong to branch 4 and are again related to human strains present in West Africa (Honap *et al.*, 2018).

However generally, the prevalence of natural leprosy infection in non-human primates is thought to be low, based on negative results for example from a proactive screening of buccal swabs of 41 wild ring-tailed lemurs (*Lemur catta*) from Madagascar and 22 wild chimpanzees from Uganda (Honap *et al.*, 2018).

In experiments 80% of sooty mangabey monkeys (24/36) inoculated with leprosy bacilli developed leprosy. Experimental intravenous and intradermal infection was also successful in rhesus macaque (*Macaca mulatta*, 7/34) and African green monkeys (*Cercopithecus aethiops*, 15/19), while squirrel monkeys (*Saimiri sciureus*) were resistant to the infection (Gormus *et al.*, 1988; Meyers *et al.*, 1991).

Twenty-four cynomolgus monkeys experimentally infected with *M. leprae* showed negligible susceptibility to the disease. Four animals developed papules at the inoculation site, but then lived for 2-8 years without showing skin lesions or neurological deficits. On necropsy AFB were observed in the earlobes, scrotum, ulnar nerve and nasal mucosa. Among the *M. leprae* sources were three mangabey monkeys, two of which were also simian immunodeficiency virus (SIV) positive, human multibacillary leprosy patients and an experimentally infected armadillo. Of the four cynomolgus monkeys that became sub-clinically infected by *M. leprae* one was infected with *M. leprae* from all three sources, two with human isolates and one with a mangabey monkey isolate (Walsh *et al.*, 2012).

An experimental infection of mice and rats is only successful in thymectomised and immunosuppressed individuals, and infection usually remains limited to the footpad (Hobbs *et al.*, 1978). While these species are thus unlikely to play a major role as natural hosts, particularly the mouse footpad model is of high scientific importance for the propagation of leprosy bacilli (Lahiri *et al.*, 2011).

Recently, *M. leprae* DNA was isolated from nasal swabs from one captive margay (*Leopardus wiedii*), one wild and one captive lowland tapir (*Tapirus terrestris*), two wild capuchin monkeys (*Sapius apella*), and one wild owl monkey (*Aotus trivirgatus*) from the Mato Grosso and Pantanal region of Brazil, after collecting samples from 69 wild and captive animals belonging to 25 different species (Autorizac *et al.*, 2018). However, the strains detected were not sequenced in full and the presence of leprosy bacilli on the nasal mucosa alone cannot provide information on whether these animals were transiently colonised or actually infected by the bacterium.

Low numbers of tissue samples collected from a range of species in Brazil and screened by PCR did not contain *M. leprae* DNA. This included Ring-tailed coati (*Nasua nasua*, n= 2), skunk (*Didelphis albiventris*, n= 1), hedgehog (*Sphigurrus spinosus*, n= 1), crab eating racoon (*Procyon cancrivoros*, n= 1), restless cavy (*Cavia aperea*, n= 1), ferrets (*Gallictis vittata*, n= 2), and crab eating fox (*Cerdocyon thous*, n= 2) (Pedrini *et al*., 2010). Another species that has recently been screened for the presence of *M. leprae* DNA in Brazil using buccal swabs are marmosets (*Callithrix jacchus*, *Callithrix penicillate*, and hybrids of both; n= 98). No *M. leprae* DNA was detected in any of the samples (Housman *et al.*, 2015).

Given this evidence it appears possible that the full host spectrum of *M. leprae* and *M. lepromatosis* is still unknown, and that in some species very specific, individual factors will

sometimes make an individual susceptible to an infection with these pathogens, even if most immunocompetent members of the species are resistant in most circumstances.

Infections with AFB that were associated with skin disease resembling leprosy have been described in other species as well, but where genetic analysis was available the bacteria were identified as mycobacterial species other than *M. leprae* or *M. lepromatosis* (Hutyra, Marek and Manninger, 1938; Rojas-Espinosa and Lovik, 2001; Malik, Brien and Fyfe, 2009; Pin *et al.*, 2014).

Clinical signs and histological presentation in other species

In chimpanzees disseminated LL with swellings and nodules on face, around eyes, lips, abdomen forearms and lower legs has been described (Walsh, Meyers and Binford, 1986; Suzuki *et al.*, 2010). In mangabey monkeys clinical symptoms in line with human LL have been described. Clinical signs in this species included infiltration and thickening of facial skin, erythematous thickening of the margins of both ears, lesions on the lateral surface of the feet that can ulcerate and anaemia (Gormus *et al.*, 1988). As the number of clinical cases described is low, differential diagnosis are not widely discussed and will depend on the individual presentation observed in an animal. Histological presentations have been described as being in line with human BL-LL and LL leprosy (Meyers *et al.*, 1991; Suzuki *et al.*, 2010).

Clinical disease caused by *M. leprae* or *M. lepromatosis* in other species is not currently described.

Diagnosing leprosy in other species

The few reported cases of leprosy in primates were diagnosed using histopathological methods following the observation of suspicious skin lesions (Walsh, Meyers and Binford, 1986; Gormus *et al.*, 1988). Serological assessment of αPGL-I levels has been used successfully in a chimpanzee after clinical signs of leprosy had been identified (Suzuki *et al.*, 2010). Recently, molecular methods have been used to sequence the *M. leprae* strains present in historic primate cases and to screen, unsuccessfully, for leprosy in two wild primate populations (ring-tailed lemurs from Madagascar and chimpanzees from Uganda) (Honap *et al.*, 2018).

As mentioned before nasal swabs have enabled the detection of *M. leprae* DNA in an owl monkey, two capuchin monkeys, tow lowland tapirs and a margay (Autorizac *et al.*, 2018). However, where only this sample type is available it cannot be said whether the animal was infected with the bacillus or its upper respiratory tract only transiently colonised. Buccal swabs from marmosets did not allow the identification of any leprosy carriers (Housman *et al.*, 2015). It is not possible to say whether this is due to an absence of disease or this sample type being unsuitable for leprosy diagnostics in this species.

Leprosy as potential zoonosis

Diseases affecting wildlife species are of human concern in several respects. From a biocentric position, wildlife diseases are relevant as they may pose a threat to ecologically important or conservation sensitive species and they could be the cause of avoidable individual suffering, where they are amplified due to imbalances in ecosystems under stress. From an anthropocentric position they are relevant as they may pose a threat to domestic animal and human health, when they are caused by pathogens that can affect more than one host (Decker *et al.*, 2016). If such multi-host diseases affect humans and other animals, they are called zoonotic. Zoonoses are defined as “diseases naturally transmissible between vertebrate animals and man including those transmitted by direct contact with infected animals or carcasses, by food or water contamination, and by inhalation of infected dust” (Palmer, 2011). In laymen dictionaries the specification “vertebrate animals” is often reduced to “animals” (e.g. Cambridge Dictionary, Merriam-Webster), thus potentially creating confusion as this could imply including for example invertebrate borne diseases in the term.

Zoonotic disease research often focuses on which diseases could be transmitted TO a human, paying less attention to diseases that can be transmitted BY humans to other species. However, looking at examples for the latter, highly relevant pathogens such as methicillin-resistant Staphylococcus aureus, influenza A virus and *Mycobacterium tuberculosis* are included (Messenger, Barnes and Gray, 2014). *M. leprae* is another example.

Historic disease distribution information (Blake *et al.*, 1987; Truman, 2005), experimental data (Storrs *et al.*, 1974; Hamilton *et al.*, 2008) and genetic evidence (Truman *et al.*, 2011; Avanzi *et al.*, 2016; Benjak *et al.*, 2018; Honap *et al.*, 2018) suggest that NBA, ERS and several primates did become infected with leprosy from a human source. Some species or even just individual populations of these species then appear to be able to naturally sustain the infection, as seen in NBA and ERS (Williams and Loughry, 2012; Avanzi *et al.*, 2016). This can and is thought to have resulted in an expansion of the pathogen range along with the NBA host species range in the Southern USA (Loughry *et al.*, 2009). However, NBA are currently present in Texas, Oklahoma, Kansa, Missouri, Arkansas, Louisiana, Mississippi, Tennessee, Illinois, Kentucky, Alabama, Georgia, Florida and South Carolina (Taulman and Robbins, 2014), while human leprosy cases are reported from other states such as California, Hawaii or New York as well (Aslam *et al.*, 2019). The example of ERS shows that leprosy may remain endemic in the animal host population beyond its eradication in the local human population (Avanzi *et al.*, 2016).

Paragraph eliminated

While leprosy is likely to have been transmitted from humans to other animal hosts in the past (Schuenemann *et al.*, 2018), it is harder to tell whether, how and how frequently a

transmission from an animal host back to humans occurs. Case studies have to rely on humans reporting NBA contact, and as contacts often date back by years or decades, it is not usually possible to obtain samples from these animals anymore. Even in cases, where NBA were trapped for leprosy research and it should be assumed that these animals were tested for leprosy when first taken into captivity, information about the leprosy status of the NBA a person had contact to is usually not available (Logas and Holloway, 2019). The dynamics of leprosy transmission may also be changing over time. This could for example be caused by changes in host range overlap or when susceptibility in one host population shifts towards resistance, changing in which host intraspecies transmission can occur most effectively, or at all.

Human to human transmission continues to be the most effective route for a person to become infected with leprosy bacilli (Pedrini *et al.*, 2010; Araújo Stefani *et al.*, 2019). Reports linking human leprosy cases to NBA contact in a geographic area to which human leprosy is not endemic exist (Lumpkin III *et al.*, 1983; Domozych *et al.*, 2016), but at the same time autochthonous cases are reported from non-endemic areas where no armadillos exist or where patients cannot recall any contact to armadillos (Villada *et al.*, 2016; Rendini and Levis, 2017; Aslam *et al.*, 2019). Even attempts to link NBA meat consumption and leprosy risk have returned inconsistent results so far (Deps *et al.*, 2008; Schmitt *et al.*, 2010).

The low prevalence of leprosy in some NBA populations also means that humans can have contact with many armadillos without being exposed to leprosy bacilli. Together with the fact that no autochthonous human leprosy cases occur in the UK where an animal reservoir is present as well, and the generally low transmissibility of leprosy bacilli, this implies that while the risk of leprosy transmission from an animal source to humans exists, it is most likely very low (Truman, 2008; Sharma *et al.*, 2015; Avanzi *et al.*, 2016; HAIRS, 2016). Prolonged close contact to or sharing the environment with heavily infected and clinically diseased animals in bad socioeconomic conditions may increase the risk (daSilva *et al.*, 2018), at least for the minority of humans (5-10%) who are still susceptible to an infection with leprosy bacilli (Scollard *et al.*, 2006).

Additional sources of infection are likely to exist. This could be subclinically infected or colonised human and non-human animal hosts, which are difficult to detect, not yet identified host species or non-vertebrate environmental sources (Blake *et al.*, 1987; Araujo *et al.*, 2016). Especially in India, where no animal reservoir for leprosy is known and new cases keep emerging in certain areas without a history of prolonged contact to leprosy patients, it has been suggested that the environment also plays a role in the transmission of leprosy (Mohanty *et al.*, 2016). Both *M. leprae* DNA and RNA, as well as *M. lepromatosis* DNA have been successfully detected in the environment in areas where leprosy hosts live (Miskin, Farrimond and Head, 1999; Chilima *et al.*, 2006; Lavania *et al.*, 2008; Mohanty *et al.*, 2016; Tio-Coma *et al.*, 2019), more specifically from soil, washing and bathing places (Turankar *et*

al., 2012; Mohanty *et al.*, 2016; Tio-Coma *et al.*, 2019). Detection rates are variable and lower in areas where no active human cases reside. Interestingly, *M. leprae* DNA was still present in soil in areas where no known cases of leprosy had resided in the past 5 years (Turankar *et al.*, 2019). A majority (76.7%) of natural water sources can contain viable *M. leprae* (determined by the presence of mRNA) in areas where human leprosy is endemic (Virk *et al.*, 2017). It has been shown that *M. leprae* can survive up to 46 days in wet soil (Desikan and Sreevatsa, 1995). It has been suggested that free-living amoeba could become infected by *M. leprae*, thus allowing the pathogen to survive and remain infective in the environment for extended periods of time (Wheat *et al.*, 2014; Franco-Paredes and Rodriguez-Morales, 2016; Turankar *et al.*, 2019). AFB expressing *M. leprae*-specific PGL-1 have also been isolated from Sphagnum mosses in Norway (Rojas-Espinosa & Lovik 2001). In ticks, more precisely in the species *Amblyomma sculptum*, the transovarial transmission of viable *M. leprae* has been experimentally demonstrated. After offspring of orally infected adults fed on a rabbit, viable bacteria were present in the skin (Ferreira *et al.*, 2018). Leprosy transmission does not obviously show seasonal patterns, but may be influenced by other climatic factors such as temperature and humidity (Valois, Campos and Ignotti, 2015).

Many other diseases caused by members of the family Mycobacteriaceae are zoonotic, sapronotic (organic matter, soil and plants can be a reservoir) or both, and most share the characteristic of a wide geographic distribution with leprosy bacilli (Hubálek and Rudolf, 2011). Research into all these pathogens struggles with the complexity that comes with a wide range of hosts and additional reservoirs, where some potential sources may not be identified until an unusual emergence of disease cases stems from one of them, or there is a commitment to widespread, active surveillance. Despite being one of the longest known diseases, leprosy remains an elusive and puzzling disease, where any new discovery is hoped to finally hold the key to understanding and controlling it. Whether leprosy truly deserves to be labelled a zoonosis will depend on evidence of continued transmission events across species barriers being presented.

1.1.5. ERS in the British Isles – an endangered wildlife species

ERS are small, tree-living, terrestrial mammals belonging to the *Sciuridae* family within the order *Rodentia*. ERS have a body temperature ranging from 38 to 40°C (Bosch and Lurz, 2012), which does not drop significantly even in times of reduced activity (Dausmann *et al.*, 2013). Mortality rates within the first year of life can be as high as 75-85%. Those who survive the first six months have an average life expectancy of three to five years in the wild and occasionally up to ten years in captivity (Bosch and Lurz, 2012). The average year to year adult survival in ERS populations is ~50%. About 16% of a population are taken by predators each year (Lurz, Gurnell and Magris, 2005).

Aside from the well-documented population declines in the British Isles and Italy, ERS are common throughout their large Palaearctic range on the Eurasian continent and considered

as least concern by the International Union for Conservation of Nature (IUCN; Shar *et al.*, 2016). Beyond its native range the species has been introduced into Armenia, Azerbaijan, Georgia, Kazakhstan, Kyrgyzstan, and Saint Kitts and Nevis. Populations can flourish in coniferous forests, but also in deciduous woods, mixed forests, parks, gardens, and even just small stand of conifers. Population densities are usually about 0.1 to 1.5 individuals per hectare. At high densities behaviours like bark stripping can lead to forest damage (Shar *et al.*, 2016). ERS mainly feed on seeds, acorn, fungus, bark and sapwood, but occasionally eggs, young birds and bone are consumed (Bosch and Lurz, 2012; Shar *et al.*, 2016). Coat colours vary in different shades of red through to almost black. Albinos are rare (Madsen, 2011). ERS are in some countries (e.g. Japan) kept as pets and in others still hunted for their fur (e.g. Mongolia). Historically ERS were hunted for their meat and pelts across their range (Shar *et al.*, 2016; Inskip *et al.*, 2017). ERS are listed on Appendix III (Protected fauna species) of the Bern Convention on the Conservation of European Wildlife and Natural Habitats (Shar *et al.*, 2016).

ERS have been present in the British Isles since circa 5000-8000 BC. Populations here have always fluctuated but the recent population decline has been so steep that populations are at risk of local extinction in the short term but without management, eventual complete extinction (Madsen, 2011; Bosch and Lurz, 2012). Though road traffic and predation by pets are the most frequent causes of death for red squirrels (as reported from surveillance efforts), the lack of suitable habitat, resulting in malnutrition and starvation, fragmentation of habitat, and particularly the competition from introduced Eastern grey squirrels (*Sciurus carolinensis*, GS), in conjunction with the introduction of squirrel poxvirus significantly contribute to the decline (Meredith and Romeo, 2015). Up to 61% of healthy GS can carry the poxvirus and under these circumstances can replace ERS 20 times faster than if only direct competition occurred (Rushton *et al.*, 2006; Bruemmer *et al.*, 2010).

The main stronghold for ERS in the British Isles is Scotland, with about 120,000 individuals remaining. Populations in England (~15,000), Wales (~3,000) and Ireland (40,000) are much smaller and in many instances fragmented or limited to islands or protected areas (Gurnell, 2013). Most of the British ERS are of continental ancestry, with a recent (1960's) surge in ERS with Scandinavian origins (Hale, Lurz and Wolff, 2004).

In the United Kingdom ERS are protected under the Wildlife and Countryside Act 1981 Section 11, as amended by the Countryside and Rights of Way Act 2000 for England and Wales, and the Wildlife (Northern Ireland) Order 1985 Article 12, as well as the Wild Mammal (Protection) Act 1996, the Welfare of Animals (Northern Ireland) Act 1972, and the Animal Welfare Act 2006. This means ERS cannot be intentionally killed, injured or captured, their shelters may not be intentionally or recklessly damaged or destroyed, and they may not be possessed or sold. Exceptions for research and conservation purposes require the appropriate licences. The regulations laid out in the Wildlife and Countryside act to manage

the threat from invasive grey squirrels were reinforced in the Invasive Alien Species (Enforcement and Permitting) Order in 2019 implementing the EU Regulation (1143/2014) on invasive alien (non-native) species. A UK Species Action plan has been laid out to facilitate conservation efforts. Dedicated conservation organisations are working towards the protection of remaining ERS populations and aim for their restoration to suitable habitats within the UK, where it is possible to remove GS. Many of these projects are very successful, however, the continuing threats have not yet allowed the overall population numbers to increase significantly again (Shuttleworth, Lurz and Halliwell, 2015).

Impact of infectious disease on ERS populations

Understanding infectious diseases present in a conservation sensitive species is of particular interest, as disease risk analysis has become an integral part of reintroduction and conservation translocation efforts and threats to these species can only be managed when they are known (IUCN/SSC, 2013; McInnes, 2018). It is also important to remember that, while infectious disease may currently only be a threat to few species, their impact on already-endangered species, with few viable populations remaining, can be immense (Smith, Acevedo-Whitehouse and Pedersen, 2009). Surveillance in existing populations has and continues to provide the necessary baseline data on existing diseases and the impact they are having. It allows to identify potential threats to ERS (Duff *et al.*, 2010; LaRose *et al.*, 2010; Simpson *et al.*, 2013; Meredith and Romeo, 2015; Shuttleworth *et al.*, 2015; Blackett *et al.*, 2018). As these efforts usually rely on convenience or opportunistic sampling, they may not be representative of the wider population and may not allow the identification of all pathogens present (Meredith and Romeo, 2015). ERS are susceptible to a range of infectious agents, some of which appear to have little impact on ERS survival, while others have been linked to numerous mortality events. It has been implied since the 1930s that infectious diseases may play a role in ERS population declines (McInnes, 2018).

Table 5 summarises infective agents, the effect they are having in British ERS and control measures in place for their management. Potentially zoonotic pathogens are indicated in bold.

Looking at the data provided by surveillance efforts on the Isle of Wight, on Jersey and in Scotland changes associated with infectious disease occur in circa 35% of ERS found dead (LaRose *et al.*, 2010; Simpson *et al.*, 2013; Blackett *et al.*, 2018). This implies that they can be factor with high impact on ERS populations and makes it urgent to understand the extent to which each of them could pose a threat and how this threat could be managed. This is particularly true in populations where lowered genetic diversity and the presence of genetic traits possibly conferring lower disease resistance do occur. This is was hypothesised to be the case in ERS populations in the British Isles (Barratt *et al.*, 1999; Hale, Lurz and Wolff, 2004; Ogden *et al.*, 2005; Ballingall *et al.*, 2016; Hardouin *et al.*, 2019).

TABLE 5: INFECTIOUS AGENTS DESCRIBED IN ERS, THEIR EFFECTS AND CONTROL MEASURES IN PLACE (POTENTIAL ZOONOSES IN BOLD)

Pathogen	Effect on ERS	Control measures	Source
Squirrelpox Virus (SQPV)	Severe, erythematous, exudative dermatitis around face, feet and ventrum, ulceration of lesions and lethargy, mortality possible. Outbreaks in populations with high fatality rates (92%) occur	GS control, feeder disinfection regimes, measures to limit squirrel contact	(LaRose *et al.*, 2010; Chantrey *et al.*, 2014; Blackett, 2015; Meredith and Romeo, 2015; Fiegna *et al.*, 2016; Everest *et al.*, 2017)
Adenovirus infection (SqAdV-1)	Enteritis, splenitis, diarrhoea, sudden death, outbreaks with high mortality described in the UK and Germany; can also be non-clinical	Limit contact to potential reservoirs (GS, wood mice) and virus accumulation around feeders	(Everest *et al.*, 2014; Blackett, 2015; Meredith and Romeo, 2015; Shuttleworth *et al.*, 2015; Blackett *et al.*, 2018; Wernike *et al.*, 2018)
Rotavirus	Diarrhoea in juveniles; no effect at population level described	None specifically recommended	(Blackett, 2016)
Staphylococcus aureus MLSTCC49 luk M	Fatal exudative dermatitis (FED), necrotising lingual and laryngopharyngeal ulceration, inhalation pneumonia; can affect significant proportion (15%) of ERS sampled from a population	Good feeder hygiene to reduce transmission risk	(Simpson *et al.*, 2013; Meredith and Romeo, 2015; Shuttleworth *et al.*, 2015; Blackett *et al.*, 2018)
Staphylococcus sciuri	Secondary infection of wounds or pox lesions; no negative effect at population level described	None specifically recommended	(Duff *et al.*, 2010)
M. leprae/M. lepromatosis	Bulbous skin lesions in individual ERS, can be non-clinical; effect at population level unknown	None specifically recommended	(Avanzi *et al.*, 2016)
Pasteurella multocida	Pneumonia, Enteritis; no effect at population level described	None specifically recommended	(Meredith and Romeo, 2015)
Bordetella bronchiseptica	Bronchopneumonia, sometimes fatal; no effect at population level described	None specifically recommended	(Simpson *et al.*, 2013; Meredith and Romeo, 2015)

Salmonella spp.	Enteritis; no effect at population level described	None specifically recommended	(Meredith and Romeo, 2015)
Pathogen	**Effect on ERS**	**Control measures**	**Source**
Borrelia burgdorferi	Non-clinical (?); no effect at population level described	None specifically recommended	(Blackett, 2016)
Campylobacter spp.	Enteritis; no effect at population level described	None specifically recommended	(Meredith and Romeo, 2015)
Yersinia enterocolitica/ pseudotuberculosis/spp.	Abscesses, wound infection, pneumonia; no effect at population level described	None specifically recommended	(Meredith and Romeo, 2015)
Erysipelothrix rhusiopathie	Non-clinical (?); no effect at population level described	None specifically recommended	(Blackett, 2016)
Francisella tularensis	Non-clinical; no effect at population level described	None specifically recommended	(Meredith and Romeo, 2015)
Leptospira spp.	Non-clinical, occasional nephritis; no effect at population level described	None specifically recommended	(Meredith and Romeo, 2015)
Capillaria hepatica	Liver lesions, mortality; no effect at population level described	None specifically recommended	(Simpson *et al.*, 2013; Meredith and Romeo, 2015)
Eimeria silvana, Eimeria sciurorum	Often non-clinical, diarrhoea and haemorrhagic enteritis possible; no effect at population level described	None specifically recommended	(Blackett, 2015; Shuttleworth *et al.*, 2015)
Toxoplasma gondii	Necrosis in liver, spleen and lungs, focal cardiomyopathia; acute fatalities can occur; can have a negative impact on populations	Limit access of ERS to areas contaminated with cat faeces	(Simpson *et al.*, 2013; Meredith and Romeo, 2015)
Hepatozoon app.	Non-clinical; no effect at population level described	None specifically recommended	(Simpson *et al.*, 2013; Meredith and Romeo, 2015)
Candida albicans	Oral candidiasis; no effect at population level described	None specifically recommended	(Simpson *et al.*, 2013)
Emmonsia crescens	Adiaspiromycosis, granulomatous reaction in the lungs; no effect at population level described	None specifically recommended	(Simpson *et al.*, 2013)
Cladosporium spp	Pulmonary phaeohyphomycosis; no effect at population level described	None specifically recommended	(Simpson *et al.*, 2013)

Assessing population health

Whether a population of wild animals is 'healthy' is difficult to determine. On the individual level the modern definition of health is not the absence of disease, but the ability to adapt and self-manage (Huber *et al.*, 2011), while population health has been defined as the "health outcome of a group of individuals, including the distribution of such outcomes within the group" (Kindig and Stoddart, 2003). For wildlife, it has been proposed to define health as the capacity to cope with biological, social and environmental changes over time, based on individual and ecosystem capacities. It has been pointed out that "wildlife health is not a biologic state but rather a dynamic human social construct based on social expectations and scientific knowledge" (Stephen, 2014). Some infectious diseases or parasites may be sustained in a population without negatively affecting it as a whole, as long as no other stressors or changes to the ecosystem disrupt the balance (Real, 1996; Cunningham, Daszak and Wood, 2017).

A range of animal related parameters can be used as indicators of the health of a population. Indicators need to be measurable in some form, they should ideally be independent from one another, reliable, readily available, unambiguous and representative of an animal's health. They can include the presence of certain pathogens, morbidity and mortality events, endo- and ectoparasite burdens observed in individuals, reproductive success, general health and well-being of individuals, or for example parameters such as weight, body condition score (BCS) or results of laboratory tests assessing organ function. In some situations where lots of information is available and certain parameters show higher suitability than others to indicate health, different parameters used may be weighed and an integrated health score calculated (Depoorter *et al.*, 2015). In wildlife, observable health outcomes such as nutritional status, reproductive success, longevity and the presence of diseases and parasites are the most frequently used indicators. Much information can be collected in this manner by observing live animals from a distance and using carcasses opportunistically found for more detailed assessment and disease and parasite screenings (Jamot, 2013). Such fairly easily collectable general parameters suitable to assess individual and population health in ERS specifically are introduced in chapter 2 (p. 43).

1.2. Research objectives

Leprosy has only been described recently (2014) in ERS. Initial research using opportunistic samples has produced a bulk of highly relevant information regarding the geographic spread of the pathogens throughout the British Isles, the specific strains present as well as on some clinical presentations and established the existence of non-clinical cases (Meredith *et al.*, 2014; Simpson *et al.*, 2015; Avanzi *et al.*, 2016; Butler *et al.*, 2017). However, in live ERS the basic characteristics of leprosy have not yet been assessed and carcasses can still provide additional information to what has currently been published.

Three aspects are usually included in the basic description of a disease: clinical presentation, pathology, and epidemiological characteristics. This piece of work aims to provide new information on all three aspects, to increase the understanding of this ancient disease in its newly discovered wildlife host. The collected information should be relevant to the formulation of an appropriate response to ERS leprosy in a species conservation context.

Currently, no information exists on the diagnosis, progression and prevalence of leprosy in live ERS, or about the effect leprosy is having on ERS populations. Efforts to identify leprosy in GS in the British Isles have been minimal and it is unknown whether leprosy occurs in ERS outside the British Isles. To address these knowledge gaps the following hypotheses are explored:

1. The same diagnostic methods used in other hosts and in ERS carcasses can be applied to live ERS under field conditions.
2. The clinical presentation of leprosy in ERS is pathognomonic and very similar between individuals.
3. Leprosy has an impact on health indicators in individual ERS.
4. Leprosy is as frequently observed in male as in female ERS.
5. A histopathological lesion spectrum similar to other host species is present in ERS.
6. Molecular and histological methods are both suited to identify leprosy in ERS.
7. Leprosy bacilli can be detected in a range of organs in ERS.
8. Lesion development and changes in clinical signs of leprous ERS can be observed within a two-year period.
9. Leprosy prevalence and morbidity is similar in two British Island populations.
10. The presence of leprosy has a negative effect on ERS population health.
11. Both leprosy bacilli are present in ERS in different locations throughout the British Isles.
12. Leprosy is exclusive to British ERS.

Chapter 2 will introduce the study sites and populations used and provide information on trapping and anaesthetic methods, as well as general information on the ERS seen in the two focus populations. Chapter 3 focusses on adapting diagnostic methods for the use in live ERS (hypothesis 1). Chapter 4 collates information on the clinical and histological presentation of leprosy in ERS collected throughout this study, including the distribution of leprosy bacilli throughout the host body (hypothesis 2-7). Chapter 5 compiles longitudinal data to illustrate the progression of leprosy in ERS under natural conditions (hypothesis 8). Chapter 6 addresses epidemiological aspects of ERS leprosy in two ways. First, the prevalence and impact of leprosy on the two focus populations is explored using health indicators (hypothesis 9-10). Secondly, active surveillance efforts are made in ERS and other squirrel populations not previously assessed for the presence of leprosy, both in the UK and in Continental Europe (hypothesis 11-12). In Chapter 7 the new information gathered

on ERS leprosy is put into context in terms of its implications for ERS conservation efforts, and the potential benefits of studying leprosy in wild ERS for other hosts, including humans.

Chapter 2: Study sites and overview of study population health

2.1. Introduction

The apparently sporadic occurrence of leprosy in ERS seen in opportunistic sampling efforts makes it difficult to understand the effects this ancient disease is having on individuals and populations. To investigate the impact leprosy is having on its conservation sensitive wildlife host, *in situ* assessment over time is necessary.

Population dynamics are usually influenced by a range of factors: habitat area, habitat quality, community composition and community interactions, mutualism, parasitism and transmissible diseases, predation, competition (Begon, Townsend and Harper, 2006). Islands offer a downscaling of these factors and therefore advantages as disease study systems. Generally, species diversity decreases with island area (Begon, Townsend and Harper, 2006), resulting in fewer species that could be involved when looking at disease dynamics in such settings. Active habitat and species conservation management on islands can create a situation in which ERS populations are relatively stable over time, except for limited and predictable fluctuations depending on annual tree seed crop quality. Unexpected reductions of populations can thus be more easily identified and their causes investigated, compared to open population systems where migration would be a major additional factor to be considered. GS are still absent from some smaller British islands, and will thus not influence ERS populations here as competitors and potential source of SQPV (Chantrey *et al.*, 2014). Therefore, island populations were chosen to study leprosy in ERS.

Two different leprosy bacilli, *M. leprae* and *M. lepromatosis* have been identified in British ERS. It was therefore decided to include two island populations in this study, for which the presence of either one or the other leprosy bacillus had previously been confirmed. There is no information currently available as to when and how exactly leprosy was introduced to British ERS populations, however, data available on the bacterial strains present suggests that the initial introduction may have occurred centuries ago (Avanzi *et al.*, 2016).

Timing of the biannual trapping sessions was chosen such as to avoid the main reproductive peaks in late spring and summer (Bosch and Lurz, 2012) and very cold and wet weather, i.e. winter.

2.1.1. Selected study sites

The field study locations for this project were Brownsea Island (BI) in England (50° 41’ 30” N, 1° 58’ 20” W), and the Isle of Arran (AR) in Scotland (55° 34’ N, 5° 15’W). Access and local support were readily provided in both locations. On both islands ERS are the only wild

squirrel species present. Edinburgh was the central location for the coordination of this project as well as site of most of the post mortem and laboratory work.

Brownsea

BI is one and a half miles long and located in Poole Harbour, Dorset, in South West England. The earliest evidence of settlement on the island dates back to the 5th-century BC (NationalTrust, 2017c). Over time the island has been used in different ways. In 1927 the private owner of the island abandoned farming and other entrepreneurial aspirations, allowed the farm animals to roam freely, and natural heath and woodland to take over the island (NationalTrust, 2017c). In 1961 the National Trust, a national heritage conservation charity, took over responsibility for the island. Today the island is famous for red squirrels, other wildlife, including seabirds, water voles, and sika deer, as well as scouting (DorsetWildlifeTrust, 2017; NationalTrust, 2017a). The northern half of the island is leased and managed by the Dorset Wildlife Trust (DorsetWildlifeTrust, 2017). Of the island's 203 hectares, 127 are covered with diverse forest habitat, featuring over 100 tree species, mostly planted from the 1700s onwards (NationalTrust, 2017d) (Figure 1). The climate on BI is mild, with average temperatures of 11-17 °C in spring and 12-20 °C in autumn (Figure 2).

FIGURE 1: BI NATURE RESERVE AND DAY VISITOR ATTRACTION (COPYRIGHT: NATIONAL TRUST)

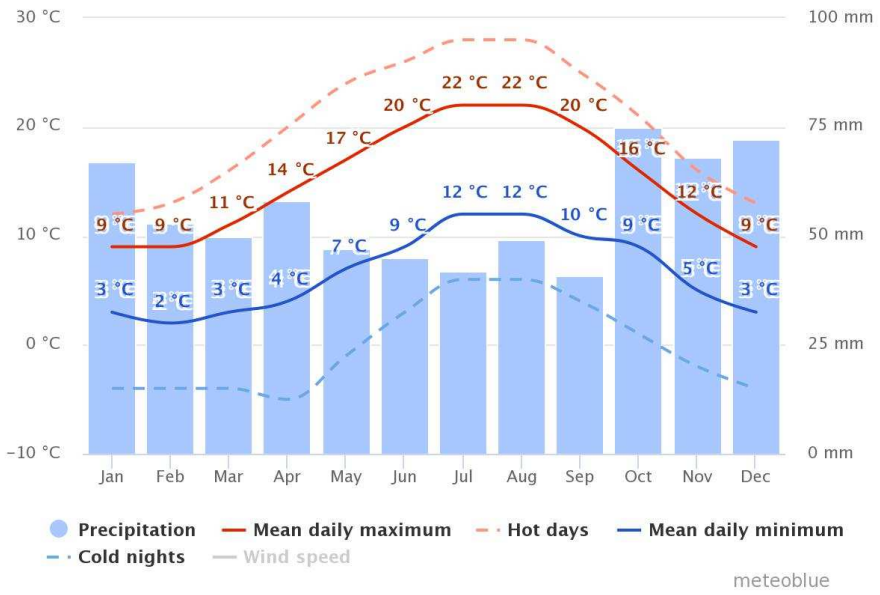


FIGURE 2: METEOBLUE CLIMATE DIAGRAM FOR BI BASED ON 30 YEARS OF HOURLY WEATHER MODEL SIMULATIONS (Meteoblue, no date a)

Arran

AR is Scotland's seventh largest island (432 km^2/43200 hectares), located on the west coast in the Firth of Clyde. It is part of the North Ayrshire council area. The woodland on the island is fragmented, with a mixture of high and low connectivity, dominated by coniferous trees and covering about 6803 hectares of the island, of which roughly 87% were seed producing in 2014 (Macpherson, 2014). The main tree species are Sitka spruce, pine, and larch (Meredith, Gurnell and Lurz, 2014). The woodlands on the eastern side of AR form a contiguous system with those in the south-central area of the island, while the connectivity is low on the western side of the island (Lurz, 2012). Roads running around and through the island can be a danger to squirrels moving around the island. Forest free areas in the hills limit squirrel movement across the island (Figure 3). Average temperatures on AR are 6-11 °C in spring and 6-12 °C in autumn (Figure 4).

FIGURE 3: TOPOGRAPHIC MAP OF AR (COPYRIGHT: ORDNANCE SURVEY, OS OPENDATA)

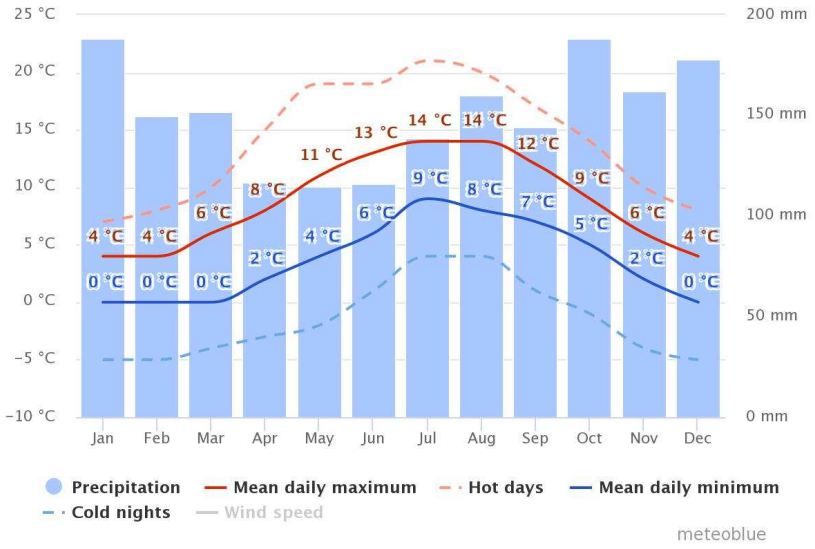


FIGURE 4: METEOBLUE CLIMATE DIAGRAM FOR AR BASED ON 30 YEARS OF HOURLY WEATHER MODEL SIMULATIONS (Meteoblue, no date b)

2.1.2. Local ERS populations

Local support efforts for ERS are similar for both islands, mainly through environmental management, but also with some provision of feeding stations or nesting opportunities. Feeding stations are used on both islands within our trapping areas to make squirrels more visible to visitors and engage the public in the protection of the species. Such wildlife hot spots are increasing the risk of infection for a range of species and pathogens (Rushton *et*

al., 2000; Adelman *et al.*, 2015). It cannot be said at this point if this is true for leprosy as well, as transmission within the ERS population is too poorly understood.

Brownsea

ERS are believed to have been introduced to BI no earlier than the 18th century (Crispin, 1979). The population is estimated to consist of about 200 individuals, with numbers varying between years depending on food availability (NationalTrust, 2017b). First written reports of clinical signs suggestive of leprosy in the population date back to the 1970s (Banks, 1971; Crispin, 1979). The population is not exposed to any specific current threats, but is limited in growth by the carrying capacity of the suitable habitat and the size of the island.

ERS on BI have been found to be infected with *M. leprae*. The disease appears to have been endemic to the island for a prolonged time and it presents with a range of clinical signs, from clinically unsuspicious to severe, pathognomonic lesions. Avanzi *et al.* (2016) found eight out of 25 (32%) *M. leprae* positive ERS (determined by detecting mycobacterial DNA in tissues) from BI to have obvious clinical signs of leprosy.

The BI ERS population was chosen as it appears to have the highest known prevalence of leprosy in the British Isles, and is one of the few places in which clinical cases of squirrel leprosy are regularly observed (Avanzi *et al*., 2016). BI is therefore an ideal study site to investigate the effects of leprosy as an endemic disease in an ERS population. However, this potentially atypically high prevalence and the fact that only *M. leprae* has been isolated from squirrels on this island (Avanzi *et al.*, 2016) may limit applicability of information collected here to other populations.

Arran

ERS have been introduced to AR from other populations, in this case most likely from the European continent. Introductions took place in the late 1930s with a second release suspected in the 1950s (Lurz, 2012). Today they present a system of linked sub-populations, which are largely in good health, and vary in numbers with annual food availability (Meredith, Gurnell and Lurz, 2014). About 400 to 1800 red squirrels can be supported by the local ecosystem, depending on tree seed crop year (Meredith, Gurnell and Lurz, 2014). For the purposes of this thesis an estimated mean population size of 1100 ERS is used. ERS are present all around the island (Lurz, 2012). The population is under no imminent conservation threat and is being managed to further increase and stabilise its numbers (Meredith, Gurnell and Lurz, 2014).

Earlier modelling studies investigating the potential effects of disease introduction on ERS on AR have assumed the town of Brodick - as the islands main harbour - to be the most likely point of introduction (Macpherson, 2014). Predictions from this study conclude that a chronic disease which is transmitted slowly, characteristics met by leprosy, would become endemic on AR following introduction to the ERS population (Macpherson, 2014).

In 2016 tissues from one out of ten ERS (10%) tested from AR were positive for the presence of *M. lepromatosis* DNA. The animal did not show clinical signs of leprosy (Avanzi *et al.*, 2016). However, members of the public have reported seeing squirrels with clinical signs of leprosy on AR.

It was assumed possible to gather information on the prevalence and effects of *M. lepromatosis* in an ERS population here that could serve as a small-scale model for the wider situation on mainland Britain and Ireland. It was also hoped that with intensified carcass collection and trapping efforts, collection of clinically diseased carcasses would enable comparison of the histological presentation of the disease caused by *M. lepromatosis* with that caused by *M. leprae* in ERS on BI.

2.1.3. Licensing

All procedures carried out in this project were approved by the Animal Welfare and Ethical Review Body (AWERB) at the University of Edinburgh (UoE) and undertaken under the appropriate Home Office Licences (PPL70/9023; PIL I3A4168A8 (AM); PIL I39FBB99D (AKS)), and additional licences by Natural England (2016-24517-SCI-SCI; 2018-36360-SCI-SCI) and Scottish Natural Heritage (90896).

Trapping, anaesthesia and general health assessment techniques for live animals and post mortem assessments for carcasses were consistent throughout the project and are presented in this chapter, along with background information on the ERS assessed in the two focus populations of this project.

2.2. Methods

2.2.1. Live trapping and general anaesthesia

Trapping

Red squirrels were trapped systematically twice a year, with each trapping session consisting of one preparation day, three trapping days, and one clean-up day. The first time an ERS was seen it was microchipped. Local members of the National Trust for Scotland and the Forestry Commission (AR), and the National Trust and Dorset Wildlife Trust (BI), placed the traps (Albion Manufacturing© Mink/Squirrel Trap Type – Albi 079 (079S, 034)) in pre-agreed locations 1-2 weeks before the planned trapping session and pre-baited with the traps fixed in an open-position.

On BI 21 non-customised traps were used. The fields outlined in yellow in the Google satellite image below (Figure 5) indicate trapping areas. Most traps (n=15) were placed in the larger area around the “Villa” in the part of the island managed by the Dorset Wildlife Trust. The remaining six traps were placed around National Trust’s “Rose Cottage”.

FIGURE 5: SATELLITE OVERVIEW OF BI. AREAS IN WHICH SQUIRREL TRAPS WERE PLACED ARE CIRCLED IN YELLOW (IMAGE SCREENSHOT VIEW OF GOOGLE MAP).

On AR traps were customised by fitting a wooden floor and a peanut reservoir. Fifteen traps were used in spring 2017. An additional 10 traps were bought following that session and worn-down traps replaced, so that in the following sessions a total of 16 customised traps were used. Exact trap locations varied slightly from session to session depending on current ERS sightings, forestry operations (thinning and felling) and risk of interference from other wildlife, particularly badgers. They were always within the large area indicated in yellow in the satellite image below. The smaller yellow square indicates the location of Brodick Castle grounds in which squirrel feeding stations exist and where traps were placed at highest density following very good trapping success rates (Figure 6).

FIGURE 6: SATELLITE VIEW OF THE SEGMENT OF AR IN WHICH TRAPS WERE PLACED. THE SMALL YELLOW SQUARE MARKS BRODICK CASTLE GROUNDS, THE LARGER YELLOW AREA INDICATES THE FULL EXTENT OF THE TRAPPING AREAL (IMAGE SCREENSHOT VIEW OF GOOGLE MAP).

At the beginning of each trip the traps were checked by the research team. If deemed necessary for success and safety purposes, they were slightly relocated, but kept close enough to the pre-baiting location to not decrease chances of successful trapping. On each trapping day the traps were set at dawn and regularly checked thereafter. On BI check intervals were between 30 min to 1 h, while they were closer to 1 h on AR.

Once an ERS was trapped, the trap was covered with a blanket and transported by hand or by vehicle to the designated processing area established for each session. Depending on a day's working speed, cut-off points at which all traps were closed were determined. This avoided excessive waiting times for squirrels between capture, processing and release, and releasing squirrels outside their normal activity hours, i.e. after sundown. Animals which did not calm down within a few minutes of being recovered were taken back to the trapping site and immediately released without attempting anaesthesia and sampling, as the higher stress levels in these animals were deemed to increase anaesthetic risk and to have a negative effect on animal welfare. Peanuts and apple slices were offered to the squirrels while they awaited anaesthesia and during recovery.

Predicted size of the sub-population covered by trapping efforts

Mark-recapture information can be used to estimate population size where the estimated number (N) of individuals in the population is calculated by multiplying the number of individuals captured and marked (M) with the total number of animals captured the second time (C) and dividing the result by the number of individuals recaptured (R).

$$N = (M*C)/R$$

In the current study setup, trap placement was confined to certain areas of both islands. Mark-recapture data can therefore not provide information on the total size of the population of ERS on BI and AR. It is however of interest to know which proportion of this total population was within the reach of the assessments by trapping just in the chosen focus areas. This allowed to fully appreciate how this approach may limit the ability to extrapolate from the current data to the populations as a whole.

Local population size estimates were therefore calculated for BI and AR, using sessions six months apart, allowing for four calculations for BI and three for AR. The average local population size was also calculated from these individual values. All animals seen in a marking session were treated as newly marked for calculation and only those marked in the immediately previous session counted as returns for calculation.

Anaesthesia

All animals were visually assessed for fitness for anaesthesia while in the trap by carefully lifting the corner of the cover. Conditions that would have excluded an animal from anaesthesia were heavy pregnancy or immediately obvious active lactation. In accordance

with the Home Office licence, animals with acute injuries were anaesthetised and assessed by a veterinary surgeon.

All animals were transferred from the trap into a bottle shaped clear plastic induction chamber using hessian sacks for stress reduced transferral and avoidance of direct handling. Once in the induction chamber, the chamber was immediately covered to keep the animal calm, and connected via an Ayes's T-piece circuit to a Stinger backpack anaesthetic gas machine (Darvallvet). Before and at the end of each anaesthesia the circuit and system were flushed with oxygen. Induction was achieved using 0.6-1.5 l/min oxygen and 5% isoflurane, closely monitoring the reaction of each individual.

Once all proprioceptive reflexes were lost, which usually occurred within the 5-10 minutes previously described for inhalant induction of rodents (Heard, 2014), the animals were taken from the induction chamber and a mask fitted. Anaesthesia was maintained on 0.6 to 1 l/min oxygen and 1.8-3% isoflurane, closely monitoring the respiratory rate and anaesthetic depth of each individual, aiming for a light anaesthetic plane sufficient for sample collection. During the anaesthetic period the animals were placed on a heat pad to maintain body temperature. In accordance with the Home Office licence, depending on individual findings during the health assessment, one-off veterinary treatment was provided if judged to be necessary or euthanasia was conducted under general anaesthesia. The latter was only considered when in the best interest of an individual animal's welfare, for example if animals were severely affected by leprosy and/or other diseases, or had severe injuries that could not be resolved in a one-off treatment.

Once all sampling was completed the isoflurane was turned off and pure oxygen was administered for a minute (or until ERS began to move). Each ERS was then transferred back into the trap it had been caught in, providing incontinence pads for insulation, covered and placed close to a heat source until fully recovered. Each ERS was released close to its capture site.

Equipment management

To avoid becoming a disease vector when moving between the two populations, single use equipment was employed as much as possible. Equipment that needed to be reused was cleaned thoroughly with disinfectants approved in local SOPs at the time of each trip. Equipment was stored for at least one week in a dry, clean storage following disinfection before being moved to the next work site. Fabric items, such as hessian bags and blankets were washed with disinfectant and exposed to sunlight. Traps were not moved between the islands but disinfected and stored in secure locations between sampling sessions.

2.2.2. General health assessment in live animals

A visual health check following a defined protocol (Appendix I, p. 224) was carried out for all squirrels trapped throughout this project and any abnormalities were noted. Health was

assessed each time an animal was trapped to provide an overview of the health status of each squirrel population by sampling group.

Age, sex and reproductive status

To determine a squirrel's age, the crown-rump length was measured with a ruler from the cranial base of the ear to the base of the tail, and the shin length of the left shin measured with callipers. Body weight was determined using a 600g capacity spring weighing scale (Pesola® Light-Line). Additionally, the maturity of the coat was noted. This information was combined to group the ERS into one of three age classes (juvenile, subadult, adult) as detailed in Table 6.

TABLE 6: AGE GROUPING PARAMETERS FOR ERS

Parameter	Juvenile	Subadult	Adult
Body weight	<160g	<280g	>280g
Crown-rump length	<110mm	<160mm	>160mm
Shin length	<58mm	59-66mm	>66mm
Maturity coat	Immature to almost mature	Mature	Mature
Maturity reproductive organs	Inactive	Inactive or beginning to be active	Active or signs of previous activity

More sophisticated ageing of adult squirrels is only possible in fresh carcasses, where eye lens weight, bones and teeth can offer additional information (Bosch and Lurz, 2012). In live squirrels it is only possible to say that adult squirrels are most likely born before the year in which they are seen. In females, bald circles around the teats can indicate that they have suckled young before. If this is observed in a not currently lactating or pregnant female in early spring, it is likely that it raised young the year before, i.e. is at least in the second year of adulthood/third year of life.

The sex of each ERS was noted along with its reproductive status. For males the reproductive categories used were 1) abdominal testes indicating reproductive inactivity, 2) scrotal testes usually present shortly before/after and during the breeding season, and 3) scrotal pigment usually only present in sexually mature, reproductively active ERS (Bosch and Lurz, 2012). Females were grouped as either 1) inactive, 2) in oestrus, when a mild swelling of the vulva was present and the season appropriate, 3) pregnant, when signs of pregnancy like prominent but not yet lactating mammary glands, or palpable amniotic sacs or foetuses in the uterus were observed, or 4) lactating when brief stimulation of a mammary segment resulted in milk flow (Bosch and Lurz, 2012).

Body condition score (BCS) and weight

The BCS was established by palpating the muscle and fat cover in the lumbar area and along the caudal thoracic, lumbar and sacral spine. The BCS is an easy, subjective method to assess body tissue reserves. It is independent from size and weight (Sakaguchi, 2009).

The four category body condition scoring system previously described for squirrels (LaRose *et al.*, 2010) was used as detailed in Table 7. The weight determined during the ageing of squirrels is presented along with the BCS.

TABLE 7: BCS CATEGORIES FOR ERS

Body condition	Description
Emaciated	No fat and very little muscle cover, with strongly protruding, visible bones
Thin	Little fat and muscle cover and the bone structure can be easily felt but is not visible (physiological at the end of lactation)
Normal	Bone structure is just palpable when applying light pressure to the muscle/fat cover
Fat	Impossible to distinguish hip bones and spinous processes even with pressure

General health status (GHS)

To assess the influence leprosy has on an ERS, it is relevant to assess their health independent of their leprosy status. This can provide information on whether leprous ERS are likely to suffer co-morbidities or if they can fare equally well as ERS not affected by the disease.

For the purpose of this project a GHS ignoring leprosy lesions was created. Based on observations made during the health check each squirrel was sorted into one of six general health categories:

- 1= in good health
- 2= in good health, minor injury that is likely to heal without complication or has already healed
- 3= acutely unwell, improvement likely
- 4= acutely unwell, improvement unlikely
- 5= chronically unwell, able to cope
- 6= chronically unwell, unable to cope.

Causes of observed conditions were not followed up diagnostically, due to financial and time restrictions within this project. Healed punch biopsy sites were noted but not included as old injuries, as they are of known iatrogenic origin and part of this study.

Outside this project leprous ERS should always be considered as chronically diseased, either coping with the disease or not. For the purpose of this study the leprosy status of individual ERS and populations will be described and assessed separately in the following chapters, only the number of squirrels seen with lesions will be mentioned at the end of the GHS sections of this chapter.

2.2.3. Carcass collection and post mortem assessment

Local rangers and volunteers, members of the Forestry Commission and general public collected ERS carcasses in the wild between 2015 and 2018 and made these available for this study. Some of these were in good condition (road kills), while others were incomplete (prey) or severely desiccated (discovered during annual nest box checks). In addition, the carcasses of three ERS euthanised during field work on BI (as required by the Home Office licence for animals with severely impaired welfare/unfit for release back into the wild) and of three ERS that died of sudden cardiac arrest under general anaesthesia on AR were included. All carcasses were stored frozen (-20°C) from the time of collection until post mortem examination. The post mortem (PM) examination was carried out with relevant risk management measures in place, following 24 hours of thawing of carcasses at room temperature at the UoE. The PM protocol collected information in a manner that allowed direct comparison of carcass data to that collected during live sampling (Appendix II, p. 225).

Data analysis

Information was summarised using Microsoft Excel® and R (https://www.r-project.org/). Analysis of the collected data is purely descriptive to provide a population health background for the remainder of this work. Results for general health and post mortem assessment are initially given separately for each island, but discussed together.

2.3. Results

2.3.1. Live trapping and general anaesthesia

Trapping

A total of 127 ERS were trapped for assessment across the five sessions completed on BI (1= autumn 2016, 2= spring 2017, 3= autumn 2017, 4= spring 2018, 5= autumn 2018). Only one ERS was released without attempting anaesthesia. All other squirrels appeared to settle and did consume the peanuts and apple slices offered. Across all session a total of 73 re-trapping events of ERS within the same session occurred, in which the ERS were immediately released. Traps were usually closed by noon, as squirrels very readily entered in the morning. On several occasions ERS were observed searching for peanuts outside a trap that already contained an ERS.

On AR a total of 62 trapping events with full assessment occurred across four sessions (numbering of sampling sessions followed the system established on BI, starting with 2). Re-trapping of the same ERS within a sampling session occurred only six times on this island. ERS appeared to settle calmly in the traps and were consuming the food offered.

Size of the sub-population realistically covered by our trapping efforts

The average local sub-population size in the trapping area was 54 ERS on BI, i.e. about a quarter of the estimated total island population. For AR it was 120 squirrels, i.e. 1/10 of the

total estimated average population. The estimate varied between mark-recapture pairs (Table 8).

TABLE 8: LOCAL SUB-POPULATION ESTIMATES FOR BI AND AR BASED ON THE DIFFERENT TRAPPING SESSIONS.

Marking session	Recapture session	Pop. Estimate BI	Pop. Estimate AR
Autumn 2016	Spring 2017	52	NA
Spring 2017	Autumn 2017	43	102
Autumn 2017	Spring 2018	56	84
Spring 2018	Autumn 2018	66	175

Anaesthesia

A total of 188 general anaesthetic procedures were completed throughout this study. Three animals, all from AR, did not recover from anaesthesia, representing an anaesthetic mortality of 1.6%. In all three ERS underlying pathology was detected during necropsy and attributable as increased anaesthetic risk factor (Table 9).

TABLE 9: AGE, SEX AND REPRODUCTIVE STATUS OF SQUIRRELS THAT DIED OF SUDDEN CARDIAC ARREST UNDER GENERAL ANAESTHESIA AND CHANGES OBSERVED AT POST MORTEM EXAMINATION

Animal	Age	Gender	Reproductive status	PM observations
AR029_18	adult	female	pregnant	Dilated right heart, most of lung tissue dark red and non-floating, liver dark red, fibrotic inguinal cyst
AR042_18	adult	female	inactive	Majority of lung tissue dark red colour, firm consistency & non-floating, liver enlarged & dark red
AR051_18	adult	male	Abdominal testis	Dilated right heart, some lung tissue dark red and non-floating

2.3.2. General health assessment in live animals

Brownsea

A total of 126 health assessments were completed on ERS from the BI population across the five sampling sessions.

Age, sex and reproductive status

Most trapped ERS were adults (121/126; 96%) with an overall male to female ratio of 1.14:1. No juveniles were trapped. The remaining five ERS were subadults. Trapping of subadults only occurred in autumn (Figure 7).

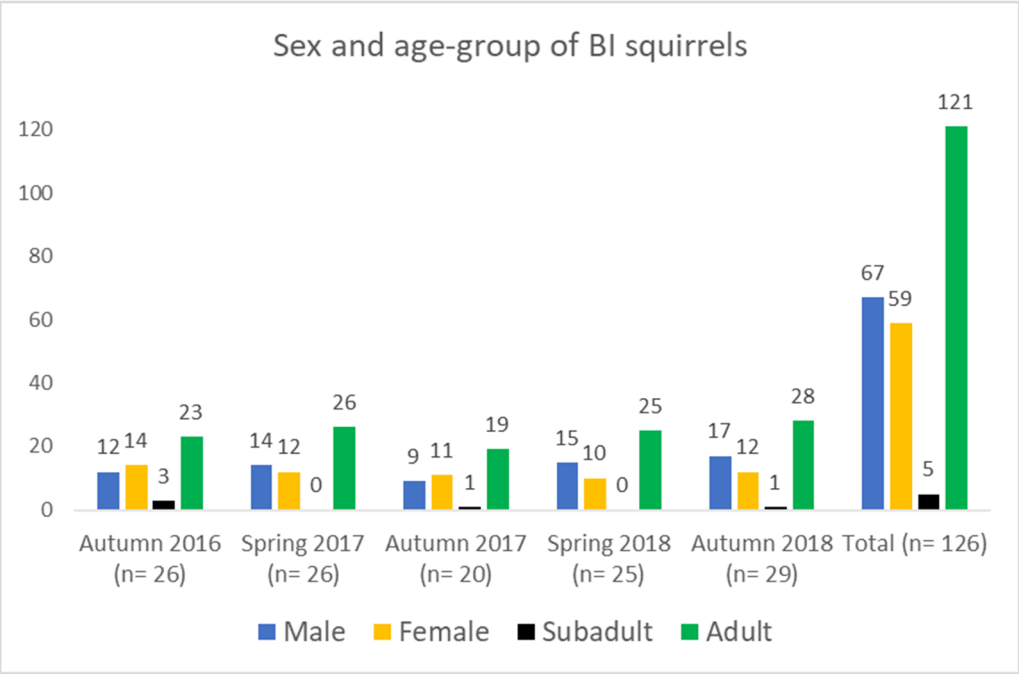


FIGURE 7: SEX AND AGE OF ERS TRAPPED ON BI PER SESSION AND IN TOTAL

The average crown-rump length measured was 169.3mm (Min= 115.0mm, Max= 185.0mm, SD= 8.4g). Shin-lengths ranged from 61.0 to 73.2mm (M= 68.8mm, SD= 2.1g).

Most adult females were reproductively inactive at the time of trapping (43/58; 74.1%). In all but one session (spring 2017) more than half of the female squirrels seen were not reproductively active (Figure 8).

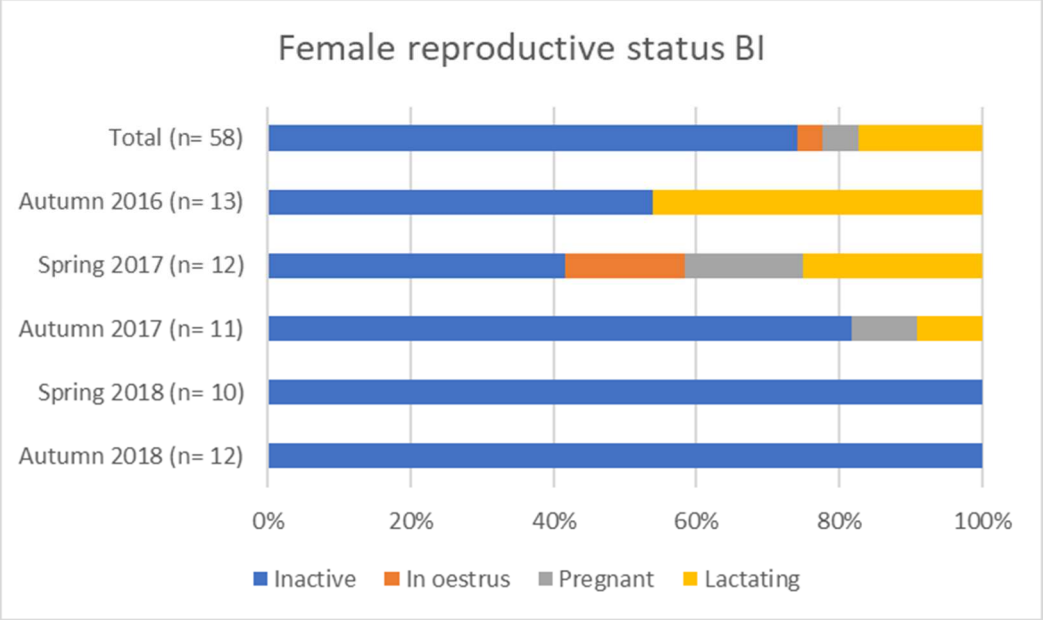


FIGURE 8: REPRODUCTIVE STATUS OF FEMALE ERS ASSESSED ON BI

In males the sexual activity status was variable. Male squirrels showing signs of reproductive activity like scrotal pigment were seen in almost all trapping sessions. More males presented

with scrotal pigment in spring than in autumn (Figure 9). The five subadult individuals were excluded from the reproductive status dataset, as they may not yet have reached sexual maturity.

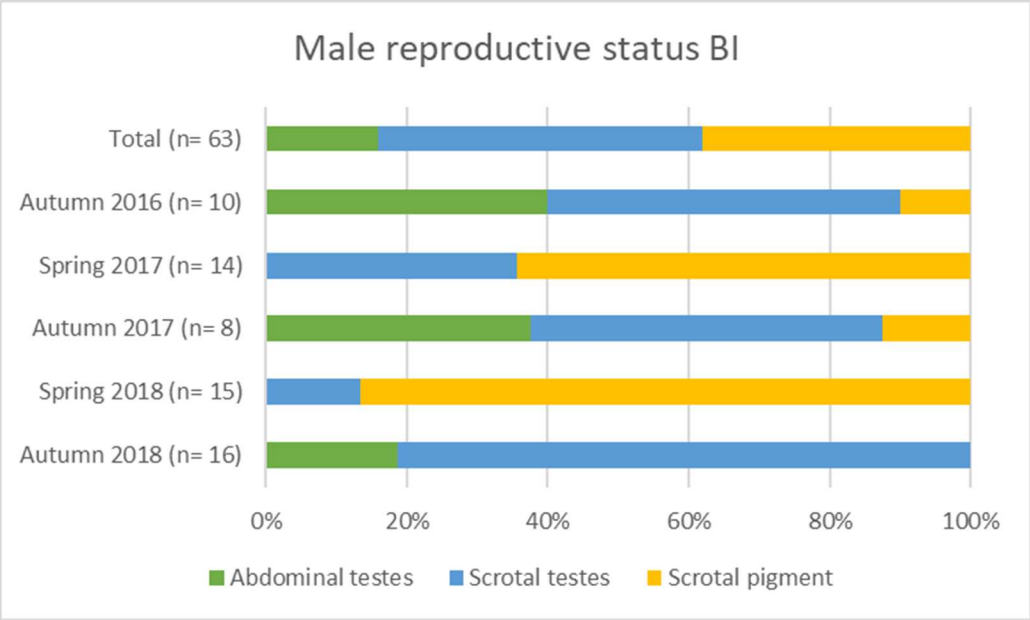


FIGURE 9: REPRODUCTIVE STATUS OF MALE ERS ASSESSED ON BI

Body condition and weight

Most animals on BI were in normal body condition at the time of the assessments (n= 79/126, 62.7%). Another 46 (36.5%) were thin. Only one animal (0.8%) was emaciated. While the proportion of normal and thin animals seen changed over time, this did not appear to reflect a seasonal variation between spring and autumn assessments (Figure 10).

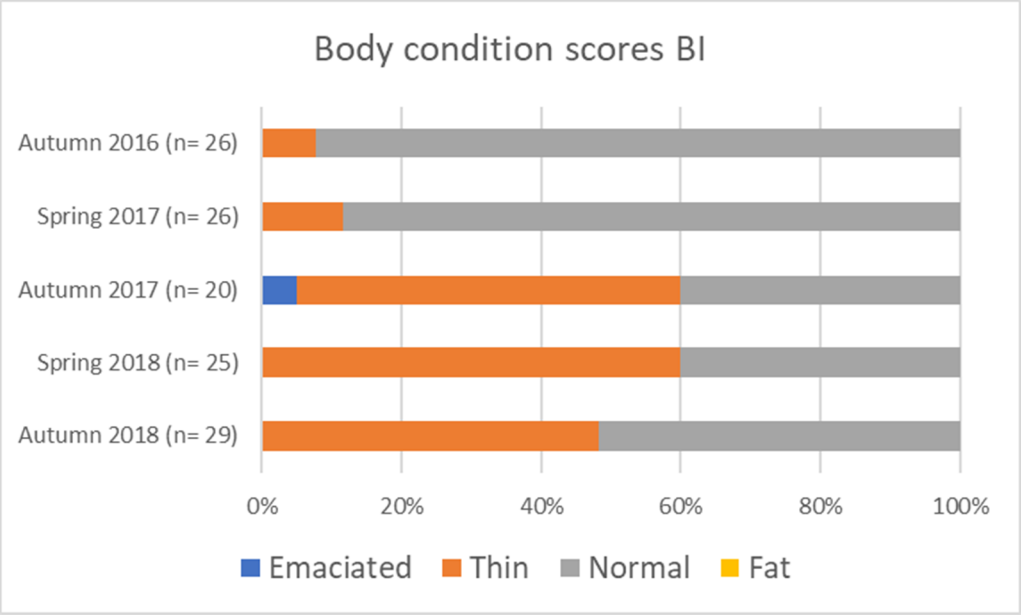


FIGURE 10: PROPORTION OF BCS OF ERS SEEN IN THE FIVE ASSESSMENT SESSIONS ON BI

The average weight of all ERS assessed on BI was 314.3g (SD= 32.9g, Min= 200.0g, Max= 435.0g). The variation of the average weight between the different sessions was minor, however some individuals weighed much more or less than the average (Figure 11).

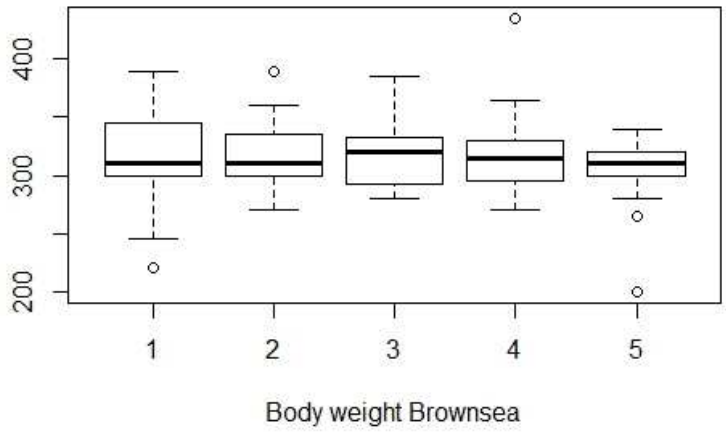


FIGURE 11: BODY WEIGHT BI ERS GIVEN IN GRAM ACROSS THE DIFFERENT SAMPLING SESSIONS.

General health status

Most BI ERS (73.8%, n= 93/126) were classified as in good health (excluding signs of leprosy). One of these animals had severe, ulcerated leprosy lesions, leading to the decision to euthanise it. Another 23.8% (n= 30/126) of the squirrels were classed as in good health with minor or healed injuries. Observed were loss of ear tissue unrelated to our sampling (n= 15), mild injuries that could have been linked to trying to escape from the trap (n= 10), old scars or scabs (n= 9), a crust on the ear (n= 1), and a missing toe (n= 1). Up to two different injury types were seen in the same animal.

One squirrel categorised as acutely unwell but likely to improve (0.8%) presented with a drained scrotal abscess, which was further treated while the animal was under general anaesthesia for assessment.

One ERS (0.8%) was classed as acutely unwell and unlikely to improve due to laboured breathing and wheezing breathing noises, indicating acute pneumonia, and severe, ulcerated leprosy lesions. Another (0.8%) was classed as chronically unwell and unable to cope for much longer, as it was suffering from a mammary abscess and intense swelling of multiple joints, with reduced range of movement, as well as severe ulcerated leprosy lesions. These two animals were euthanised in compliance with the licence conditions. Figure 12 summarises the GHS assigned to all ERS seen on BI (total), and broken down by assessment session.

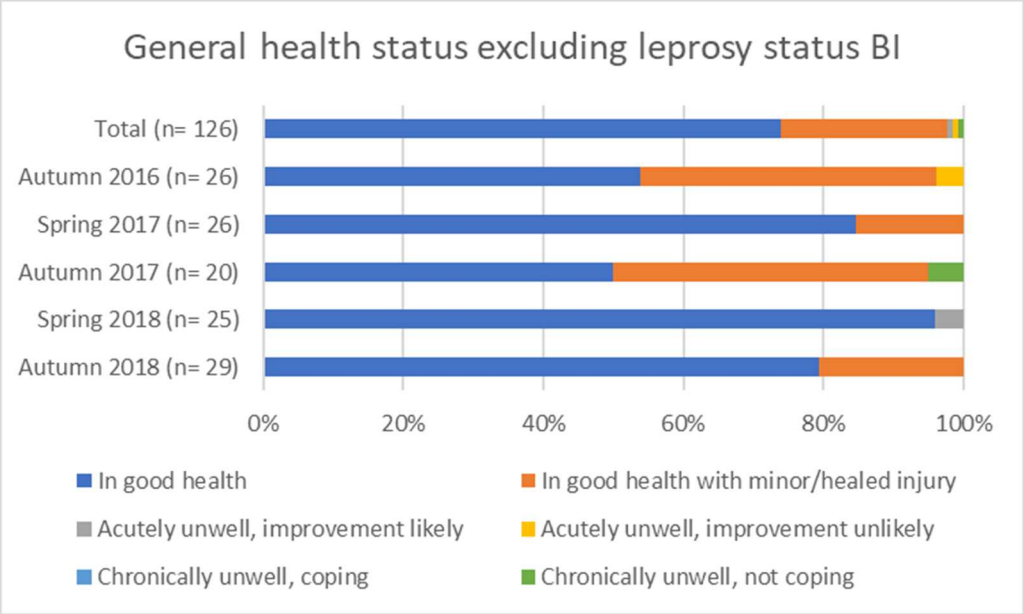


FIGURE 12: GHS OF ERS ASSESSED ON BI

Clinical skin lesions that were assumed to be due to an infection with leprosy bacilli were seen in all assessment sessions. They were seen in seven squirrels in autumn 2016, nine in spring 2017, four in autumn 2017 and spring 2018 each, and in six ERS in autumn 2018, i.e. in a total of 30 assessments across the study duration.

Ectoparasites

In one out of the 126 assessments no ectoparasites were observed on the ERS or in the induction chamber (0.8%). On 42 animals (33.3%) only ticks (*Ixodes ventalloi*, *Ixodes ricinus*, *Ixodes spp*., species information provided by the Tick Surveillance Scheme) were observed, on 13 (10.3%) only fleas (*Ceratophyllidae*, species not determined) were seen, 65 (51.6%) had ticks and fleas, four (3.2%) had ticks and harvest mites (*Trombicula autumnalis*) and on one squirrel ticks, fleas and harvest mites were seen (0.8%). Figure 13 shows the proportion of the different types of parasites seen in the different assessment sessions.

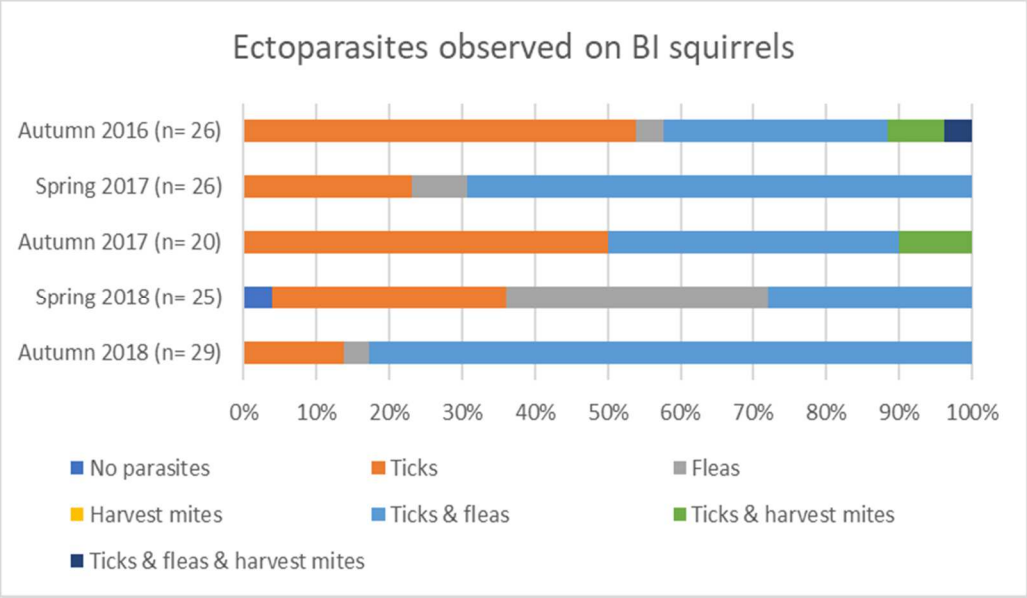


FIGURE 13: ECTOPARASITES OBSERVED ON ERS FROM BI IN THE DIFFERENT ASSESSMENT SESSIONS.

Less than five obvious ectoparasites (= intensity category 1) were noted on 49 ERS (38.9%), six to ten parasites (= intensity category 2) on 24 of the assessed ERS (19%), and on the remaining 52 ERS (41.3%) more than ten ectoparasites (= intensity category 3) were counted. More ERS with intensity category 3 were seen in autumn (Sessions 1, 3, 5) than in spring (Sessions 2, 4) assessment sessions (Figure 14).

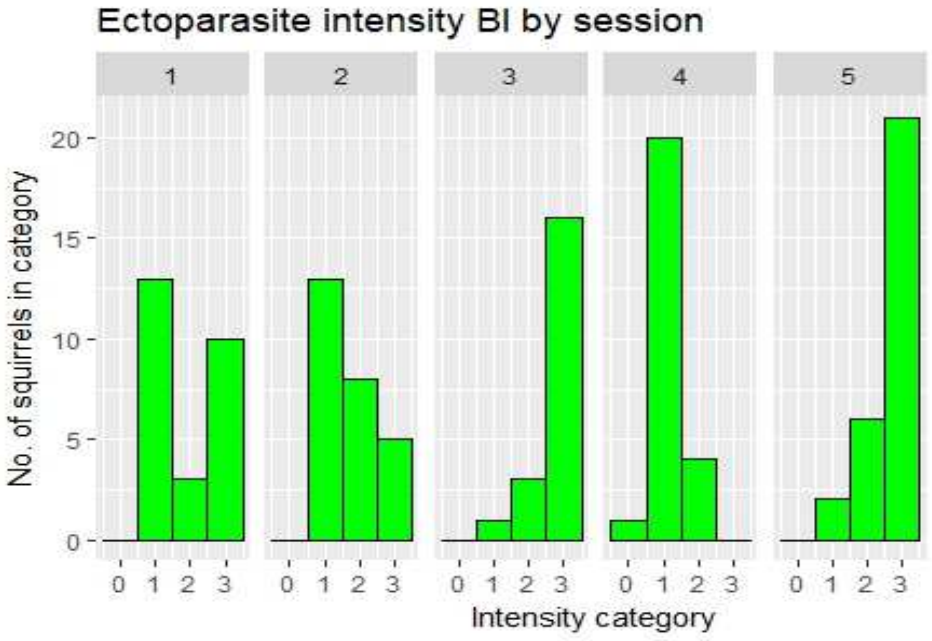


FIGURE 14: INTENSITY OF ECTOPARASITE INFESTATION OF ERS ON BI IN THE FIVE SAMPLING SESSIONS. CATEGORIES: 0= NO OBVIOUS PARASITES, 1= 1-5 OBVIOUS PARASITES, 2= 6-10 OBVIOUS PARASITES, 3= >10 OBVIOUS PARASITES.

None of the animals appeared anaemic, or showed any other signs of disease attributable to the observed parasite burdens. Ticks were often clustered around the upper body, head and ears of ERS, but were rarely present on the lower abdomen and hind legs.

Arran

On AR 62 ERS health assessments were completed across the four sampling sessions.

Age, sex and reproductive status

All squirrels assessed on this island were adults and the total number of males and females seen were almost even (32 and 30 respectively; Figure 15).

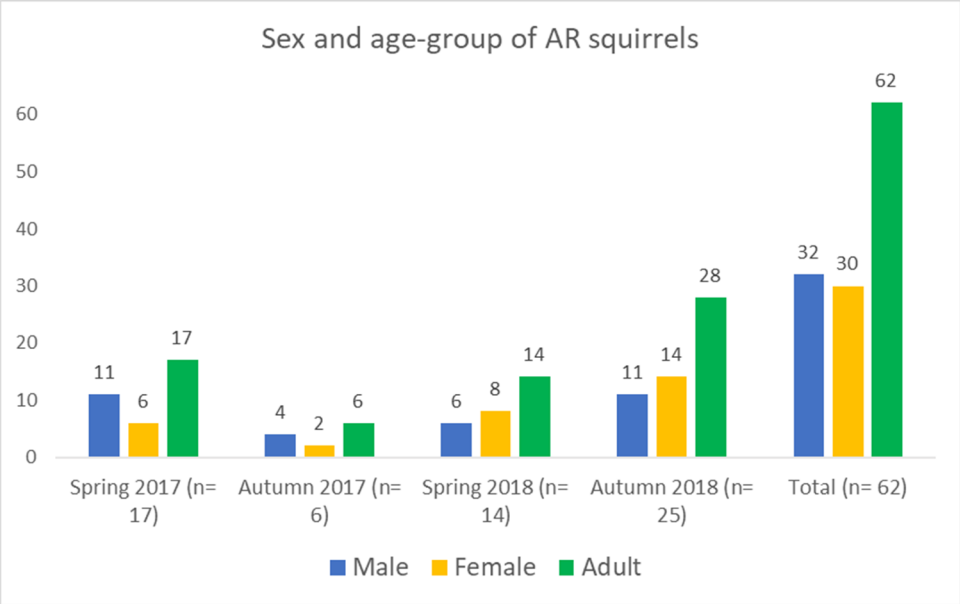


FIGURE 15: SEX AND AGE-GROUP OF THE ERS ASSESSED ON AR ACROSS ALL SAMPLING SESSIONS

The AR squirrels had an average crown-rump length of 177.2mm (SD= 7.7g, Min= 156.0mm, Max= 193.0mm). The average shin length measured was 71.3mm (SD= 2.9g, Min= 63.7mm, Max= 77.1mm).

On AR reproductively active females were only observed in spring. These were either in oestrus or pregnant. Pregnant females could only be identified by abdominal palpation, not by visual inspection. At least half of the female squirrels were reproductively inactive at the time of assessment in each session. No lactating females were trapped (Figure 16).

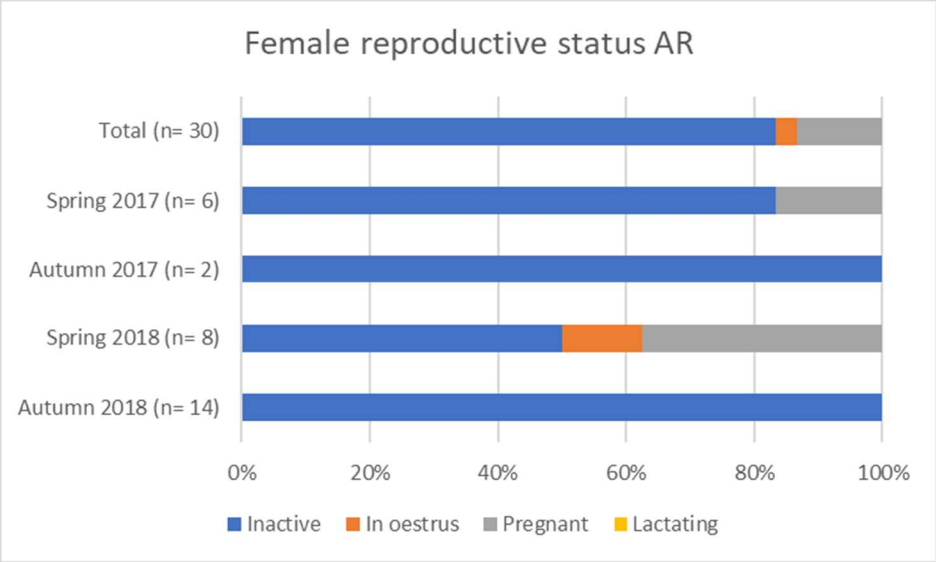


FIGURE 16: REPRODUCTIVE STATUS OF FEMALE ERS ASSESSED ON AR

Most male ERS assessed on AR in spring were reproductively active, while they were inactive in autumn. Overall, almost equal numbers of clearly reproductively active (scrotal pigment; 16/32, 50%) and inactive males (abdominal testes; 15/32, 46.9%) were seen (Figure 17).

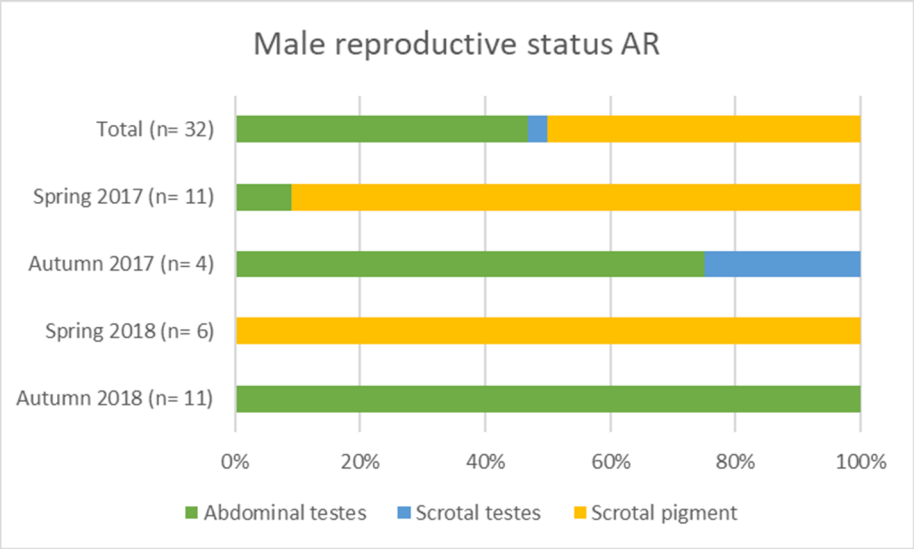


FIGURE 17: REPRODUCTIVE STATUS OF MALE ERS ASSESSED ON AR

Body condition and weight

On AR 20 ERS (32.3%) were in thin body condition at the time of assessment. The other 42 (67.7%) were in normal body condition. More thin animals were seen in spring 2018 than in the other sessions (Figure 18).

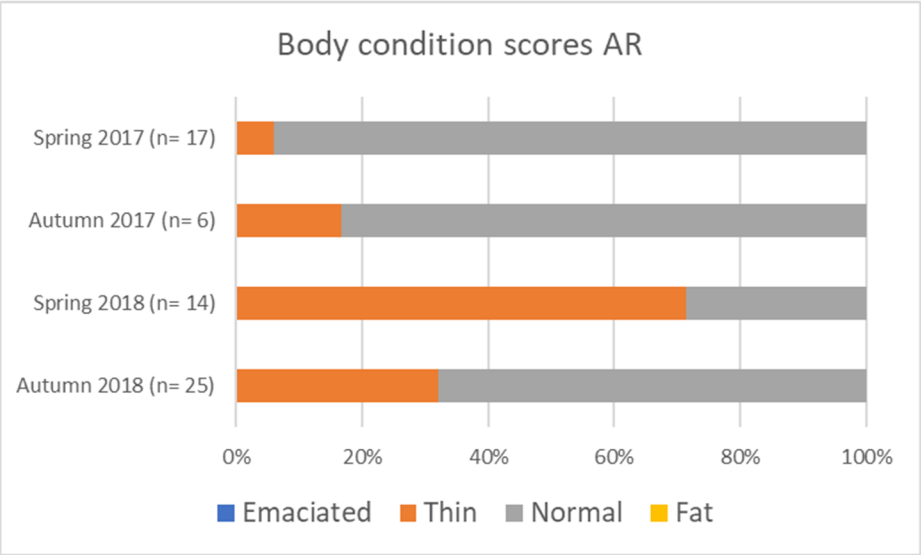


FIGURE 18: PROPORTION OF BCS OF ERS SEEN IN THE FOUR ASSESSMENT SESSIONS ON AR.

The average weight of squirrels on AR was 359g (SD 34.4g, min= 285 g, max= 430g). Average weight appeared higher in spring than in autumn (Figure 19).

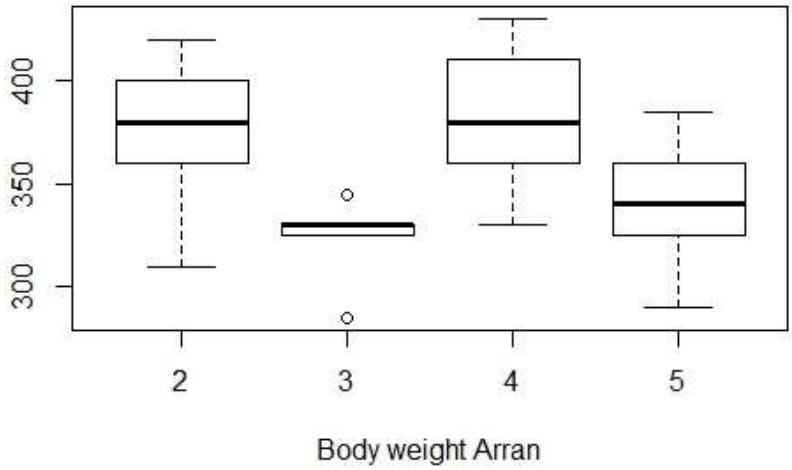


FIGURE 19: BODY WEIGHT AR ERS ACROSS SESSIONS GIVEN IN GRAM.

Due to the low number of animals trapped in autumn 2016, values from both spring and autumn sessions were combined (n= 31 each) to assess whether the weight difference between spring and autumn was statistically significant. Both sets of weights are normally distributed (Shapiro-Wilk test p= 0.496 and p= 0.377, respectively). The mean weight was 378.7g in spring and 340g in autumn. The null hypothesis that there is no difference in squirrel weight between spring and autumn can be rejected (two-sample t-test, p= 0.000003091). ERS trapped on AR had a higher average weight in spring than in autumn (Figure 20).

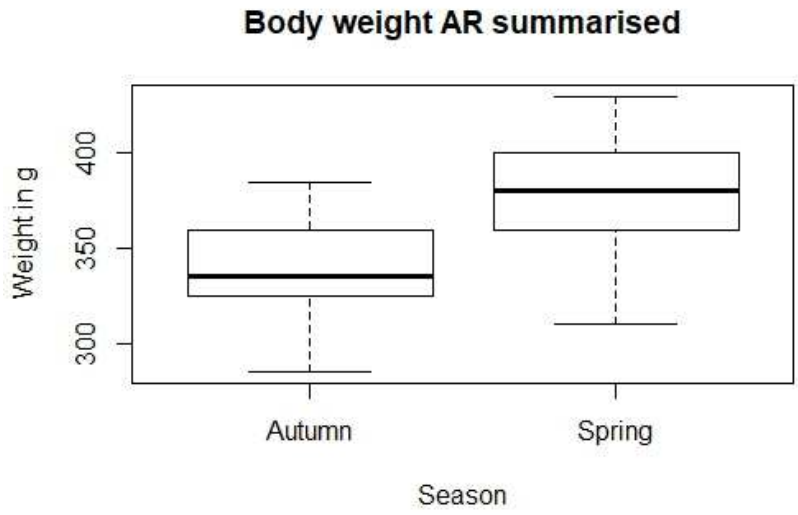


FIGURE 20: BODY WEIGHT OF AR ERS BY SEASON

General health status

The majority of squirrels trapped and assessed on AR were in good health (n= 42; 67.8%). Less than a third of the animals (n= 17, 27.4%) showed minor or healed injuries while generally being in good health. Changes observed in this group were mild trauma to the face and limbs that could have occurred while trying to find a way out of the trap (n= 8), loss of ear tissue unrelated to our sampling (n= 6), old scars indicating parasite bites or injury (n= 4), small crusts on the ear (n = 3), a healed penis injury (n= 1), a broken off lower incisor (n= 1), and hair loss and scales on the back, with signs of regrowth (n= 1). A maximum of three of these injuries were seen in the same squirrel.

Two ERS (3.2%) were classed as acutely unwell. One due to the presence of inflamed tick bites, which were deemed likely to heal with time, and the other due to an acute degloving injury to the last third of its tail that had occurred after fur got entangled inside the trap. The wound was treated and the animal released with a good prognosis, able to climb well immediately. In one ERS (1.6%) that was classed as chronically unwell and coping, an inguinal fibrotic cyst was present. This assessment only included clinical observations that could be made in the field. Otherwise, the three individuals that died under general anaesthesia and were available for full post mortem would have to be classed as chronically unwell, but coping until the additional stressor of general anaesthesia was added. However, as post mortem information is only available for this small subset of animals, it was decided to use the information that had been noted for them prior to their death, collected in the same manner as for all other animals, instead of the full information available post mortem here.

All unwell animals were seen in 2018. Overall healthy animals dominated the subpopulation of squirrels assessed on AR (Figure 21). No leprosy lesions were observed during the general health assessments.

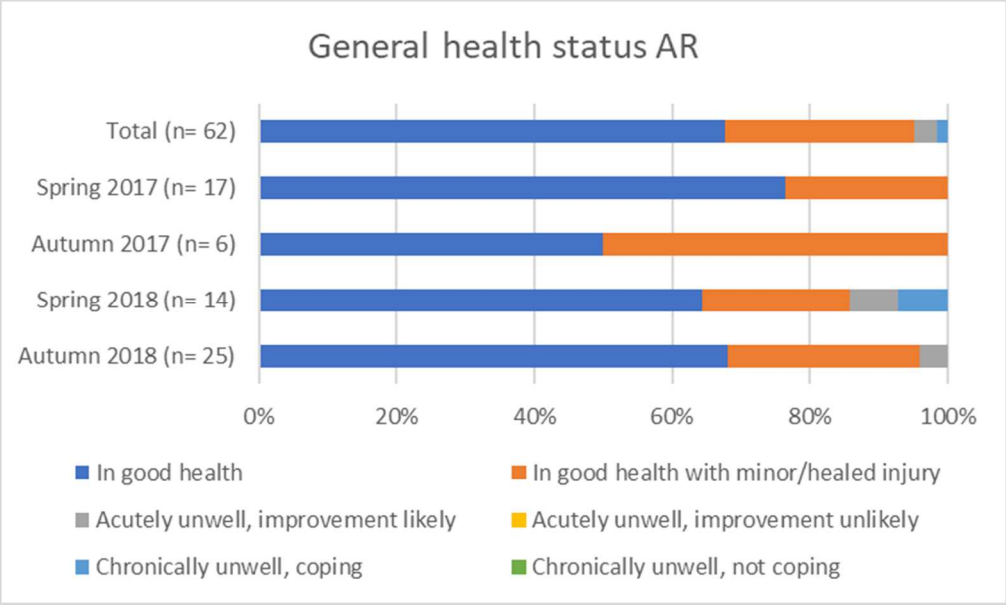


FIGURE 21: GHS OF ERS ASSESSED ON AR.

Ectoparasites

Nine animals (14.5%) had no visible ectoparasites. While both ticks and fleas were seen on the majority of ERS (n= 30, 48.4%), some had only fleas (n= 20, 32.3%) or ticks (n= 3, 4.8%). In spring no ectoparasites were found on some ERS, and very few presented with ticks, while in autumn most ERS were infested with ticks and fleas. No harvest mites were seen on AR ERS (Figure 22). The highest parasite burdens were observed on AR in autumn 2018 (Figure 23).

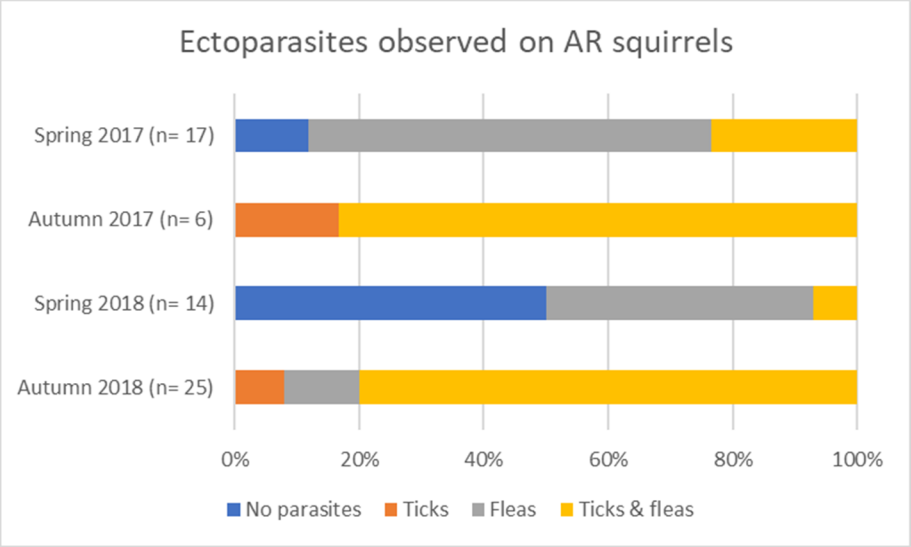


FIGURE 22: ECTOPARASITES OBSERVED ON ERS ASSESSED ON AR

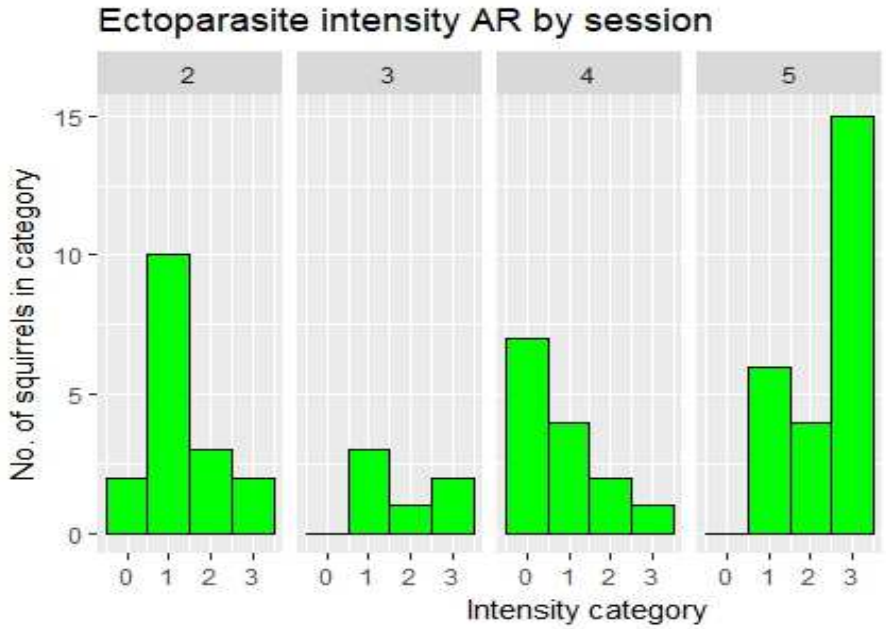


FIGURE 23: INTENSITY OF ECTOPARASITE INFESTATION OF ERS ON AR IN THE DIFFERENT SAMPLING SESSIONS. CATEGORIES: 0= NO OBVIOUS PARASITES, 1= 1-5 OBVIOUS PARASITES, 2= 6-10 OBVIOUS PARASITES, 3= >10 OBVIOUS PARASITES.

2.3.3. Carcass collection and post mortem assessment

A total of 21 carcasses were collected from BI and 29 from AR.

Brownsea

Of the carcasses collected from BI two were too desiccated for any relevant analysis, while from one other only a dried ear sample could still be collected. Therefore, post mortem examination was carried out for 18 carcasses (eight male, ten female).

Age, sex and reproductive status

Out of these 18 ERS four were juveniles, one subadult, and 13 adults. Crown-rump length could no longer be realistically determined in four carcasses due to post mortem changes. Values were thus available for nine adults, one subadult and the four juveniles. Three out of the four juveniles had a longer crown-rump length than normally expected for this age group, but for two their shin length, weight and coat were within the limits, so they were still placed here. For the third, the shin length was slightly above the expected value for juveniles as well, but the weight wasn't. As this individual was not emaciated and an investigator error of a few mm on the measurements could be possible, this ERS was overall still classed as juvenile. As a result, the average crown-rump length for carcasses in this group was 122.8mm (SD= 12.8mm). The subadult had a crown-rump length of 132mm. The adults had an average crown-rump length of 172.8mm (SD= 7mm). Shin length could be determined in all 21 carcasses originally collected. Again, values differed when animals were assessed live and post mortem by 0.8, 2 and 4.1mm. Using the post mortem values, juveniles had an

average shin length of 54mm (SD= 4.3mm), the subadult had a shin length of 56.1 mm, and adults had an average shin length of 66.2mm (SD= 3mm).

Most males (n= 6) had abdominal testicles, in one scrotal testis and in one scrotal pigment was seen. The majority of females (n= 6) were reproductively inactive, two appeared to be in oestrus, one was pregnant and one late lactating.

Body condition and weight

Assessing the BCS emaciation was noted in eight animals, while four were thin or normal and one was actually categorised as fat. In one carcass the BCS could no longer be assessed due to severe autolysis. The body weight may have been influenced by the state of the carcass in some instances. For example, some carcasses were wet after thawing and therefore heavier, others were incomplete or in advanced state of decay. Animals weighed alive and after a freeze thaw cycle were 5-20g lighter at PM examination. Eliminating values from animals whose carcasses were obviously not going to give an accurate weight (incomplete or very wet) post mortem weights were available for 15 animals. Juveniles weighed between 105 and 135g (M= 110g, SD= 16.2g). The subadult squirrel weighed only 125g and was classed as emaciated. Adults weighed between 225 and 365g (M= 308g, SD= 43.8g).

General health status

For four carcasses it was no longer possible to determine how healthy they had been ante mortem. Of the remaining 17 one (5.9%) was deemed to have been in good health. This animal was most likely killed by a bird of prey. The ERS that had been euthanised due to its severe leprosy lesions but otherwise been deemed in good health was classed as chronically unwell but able to cope following the post mortem assessment, due to presence of severe lung pathology. Another euthanised ERS (joint swelling, abscess) was classed as chronically unwell and unable to cope. The remaining 14 carcasses (82.3%) were classed as acutely unwell and unlikely to improve. For most of them acute disease was assumed to be the cause of death. In the case of the third euthanised animal it is assumed that it would have succumbed to the acute lung infection had it not been euthanised. Out of the 18 carcasses that could be assessed for the presence of leprosy lesions, seven (38.9%) showed clinical signs of leprosy, all of these were adults. For detailed post mortem findings of each carcass see appendix III (p. 226).

Ectoparasites

Some unengorged juvenile ticks were present on one carcass (5.6%) and fleas were observed on five (27.5%) carcasses. In one carcass large numbers of maggots of different developmental stages were present.

Arran

All 29 carcasses collected from AR could at least partially be assessed in a post mortem examination. The full list of observations made during the post mortem assessment can again be found in appendix III (p. 226). One ERS collected carried a microchip from having been included in a sampling session. It was run over by a car about three weeks after assessment and microchipping.

Age, sex and reproductive status

One animal collected from AR was subadult, all others were adults. Sex and breeding status could be established for 28 carcasses. Crown-rump length was measurable in 20 carcasses and ranged from 162 to 188mm (M= 172.6mm, SD= 6.8mm). Shin length was measurable in all carcasses and ranged from 65.1 to 75.2mm (M= 69.6, SD= 2.2mm). Half of them were male (n=14), the others female (n=14). Most males (64.3%) had abdominal testis, in the other five scrotal pigmentation was present. The majority of females (92.9%) were not reproductively active at the time of their death, but one was pregnant.

Body condition and weight

Body condition could only be assessed in 20 carcasses. Most (n=13, 65%) were in normal body condition while seven were classed as thin. Body weight may have been influenced as described above for BI carcasses. It ranged between 220 and 430g (M= 340.9g, SD= 35.9g).

General health status

The underlying pathology for the three animals that died of sudden cardiac arrest is detailed in Table 9 (p. 47) and appendix III (p. 226) and was likely to have contributed to their death as well as the general anaesthesia. These three ERS (10.3%) were classed as chronically unwell but coping up to the time they underwent anaesthesia. All others were classed as acutely unwell but unlikely to improve, as their acute condition resulted in their death (89.7%). One had been euthanised at a local wildlife centre after having been seen in a garden with two amputated front legs and trapped for veterinary assessment. One had puncture wounds to its chest that could imply predation and the other 24 had been run over by cars. None of the carcasses showed pathognomonic leprosy lesions.

Ectoparasites

Ectoparasites were only seen on two of the carcasses (ticks, fleas).

2.4. Discussion

2.4.1. Live trapping and general anaesthesia

Trapping

According to expert opinion (P. W. W. Lurz) the trapping efforts in this project were highly successful. High trapping success and particularly the high number of apparently ‘trap happy’ ERS on BI underline this. The lower numbers of ERS trapped on AR are likely due to

population specific factors, such as lower squirrel densities. With increased experience in later sessions trapping success increased on AR as well, likely due to more traps being strategically placed in ERS hotspots.

Areas of (artificially created) high animal densities, such as feeders or particularly crop rich habitat, which were chosen for trapping are likely to support frequent inter-animal contacts. These can be stressors, and such areas may be linked to a higher risk of disease transmission (Rushton *et al.*, 2000). This aspect will be revisited in later chapters.

While the chosen trapping regime was very successful in recruiting a large number of ERS for assessment, which was the main goal at this early stage of better understanding leprosy in ERS populations, it is likely to be biased. It only allowed to assess the subpopulation present in the trapping area (see below: Size of subpopulation realistically covered by trapping efforts). Future studies designed with different trapping strategies may be necessary to address some questions around squirrel leprosy, such as the true disease prevalence across the whole population or the presence of disease in very young live animals (Albert *et al.*, 2010). These would need a wider spread of traps and are likely to create the need to sample animals at the trapping site. This would require the transport of equipment and investigator over wider distances and would likely reduce the number of animals that could be sampled per day. Alternatively, hands off health assessment strategies tailored to leprosy involving for example camera traps or detection of pathogens in debris collected from customised, strategically placed nest boxes could allow to collect information on the whole local ERS population.

Predicted size of the subpopulation covered by trapping efforts

On both island the subpopulation likely to be present within our trapping area represented a good proportion of the total island population (1/4 for BI, 1/10 for AR). The variation between mark-recapture pairs could either be due to true variations in the population density or linked to varying trapping success due to other factors like weather, exact placement of traps et cetera.

BI is a continuous population and it is likely that the epidemiological situation is similar throughout. This could be confirmed by using different and/or additional trapping areas in future studies.

On AR the ERS population is fragmented and can vary greatly in size with food crop availability. The subpopulation sampled here was relatively small and to fully clarify the question whether truly no clinical leprosy cases are present on the island or if they are restricted to certain areas, targeted sampling in other areas of the island should be carried out.

Both subpopulations should be large enough to maintain a slow acting pathogen throughout the study period, an important factor for successful surveillance (Guberti, Stancampiano and Ferrari, 2014; Macpherson, 2014).

Anaesthesia

The anaesthetic mortality in this study (1.6%) was lower than the anaesthetic mortality rates reported for pet rats (2.01%) and wild GS (2.2%) (Brodbelt *et al.*, 2008; Parker *et al.*, 2008). The underlying chronic heart and lung conditions seen in these ERS post mortem may not have been discovered, even if a hands-on pre-anaesthetic assessment had been possible, due to the high heart and respiratory rate normal to ERS which is likely to be increased further if a wild animal was restrained for auscultation.

The stress added by handling wild ERS pre-anaesthesia is likely to increase the anaesthetic risk, and throughout the study best practice recommendations for wildlife anaesthesia were followed (Chinnadurai *et al.*, 2016) and reliance placed on expert advice (A. L. Meredith). Cardiac arrest occurred suddenly and after variable time spans. It was in all cases recognised immediately and cardiopulmonary resuscitation (CPR) initiated without success. CPR can be successfully performed in small rodents, as for example demonstrated in studies using mice and rats as models in CPR research (Papadimitriou *et al.*, 2008). However, animals in such studies are likely to not suffer from underlying conditions.

Every anaesthesia holds a risk, one that is much higher in small mammals compared to for example cats and dogs (Brodbelt *et al.*, 2008). However, without a general anaesthesia the detailed health assessment and sampling carried out in this study would not have been possible. Adverse incidence frequency was kept lower than reported for comparable species, which makes the protocol used in this study very successful and suitable for future use.

2.4.2. General health assessment in live animals

Age, sex and reproductive status

It had to be expected that given the timing and equipment used for trapping in this project mainly adults would be caught. Given previous observations, clinical leprosy appears to be a disease of adult squirrels (Meredith *et al.*, 2014; Simpson *et al.*, 2015; Avanzi *et al.*, 2016) so this bias may be an advantage for this project.

Squirrels on both islands appeared to be on average slightly smaller than the 180-280mm crown-rump length reported for ERS in the literature (Bosch and Lurz, 2012). While ERS on AR were on average slightly larger than on BI, this difference was on average less than 1cm. According to expert opinion (P. W. W. Lurz) AR ERS are larger than ERS in some mainland populations in North-west England. However, the literature does not provide enough detail on measurement methodology to know if the observed differences may just be technical. It is unlikely that a single factor is responsible for size differences in the two populations, particularly when one considers that they are likely to be of different origin (Ballingall *et al.*,

2016; Hardouin *et al.*, 2019). Chronic disease can be a cause of growth impairment (Patel, 2008). The data collected in this study may allow to assess whether there is a difference in growth of ERS affected and unaffected by leprosy within our target populations. However, many other factors, such as genetics or food availability, can also influence growth, and without having the opportunity to compare ERS populations free of leprosy living otherwise under the exact same conditions present on AR and BI, it will not be possible to assess whether the relatively smaller size of ERS in these populations is due to the presence of disease or other factors.

While not completely balanced, the sex distribution in the assessed sub-populations from both islands should permit assessment of a correlation between ERS sex and leprosy status. Fewer reproductively active ERS were encountered in the autumn assessments than in spring in both populations. Reproductive activity of ERS can occur between late December and the following August, but precise timing is influenced by factors like weather including temperature and food availability (Bosch and Lurz, 2012). More northern populations like the one on AR may be reproductively active for a shorter period than populations in warmer locations like BI. To get an initial understanding of the timescales on which leprosy progresses in squirrels and to be able to assess whether there are seasonal differences in the frequency with which cases are identified, it was necessary to trap twice a year, thus invariably ending up with one annual assessment session falling within the time during which squirrels could be reproductively active. The assessment in spring was chosen to avoid very cold and wet winter weather, which could potentially pose more intense stress on trapped animals than reproductive activity, particularly in the northern population on AR where the average winter temperature is just 4°C (Figure 4, p. 38). Pregnant females could only be identified by abdominal palpation, not by visual inspection. It was therefore not possible to avoid anaesthetising and including such animals in the current study.

Body condition and weight

On both islands the majority of animals were in normal body condition, with a smaller but again similar proportion being classed as thin. Even with one emaciated ERS seen on BI, the results do not spark immediate concern. The average weight observed was in line with the normal weight range reported for the species (202-480g) (Bosch and Lurz, 2012).

While on BI no seasonal difference in body condition and weight was observed, differences were present on AR. This could be attributed to the milder climate on BI, resulting in greater availability of food sources (Figure 2, p. 37; Figure 4, p. 38) and seems to be supported by that fact that more thin animals were seen on AR in spring 2018 than in the other sessions. This particular spring had snow in February and March, which might have resulted in reduced access to food or increased cold stress leading to a loss of body condition. Interestingly though, average body weight on AR was significantly higher in spring than in autumn. This may seem to be a contradiction, but could be explained with the inclusion of

pregnant female ERS in the spring samplings, which in AR occurred exclusively in spring. Pregnant females have a higher average body weight, but when food availability is reduced, they may still be in reduced body condition.

General health status

Leprosy has so far only been detected in dead ERS (Meredith *et al.*, 2014; Simpson *et al.*, 2015; Avanzi *et al.*, 2016; Butler *et al.*, 2017). Their death implied that they were no longer able to cope with all the factors having an impact on them, including leprosy. However, assessing the general health of individuals in two ERS populations affected by leprosy, 95% of all live squirrels assessed on both islands were “in good health” and “in good health with minor or healed injury”. If population health is seen as the sum of the health outcomes of its individuals, then this relates to both the AR and BI ERS population appearing to be by and large in good health. While some individuals may be harbouring pathogens, no single cause for obvious ill-health in large numbers of ERS in these populations was seen, if leprosy bacilli are ignored. The presence of another major disease influencing the populations thus appears unlikely. This should make the investigation into the impact leprosy bacilli have on ERS in this study viable.

On AR all unwell animals were seen in 2018, however, with data from just 2 years it is impossible to know whether this observation has any relevance. The absence of leprosy lesions on AR means that this study will not be able to compare the clinical and histological effects of *M. leprae* and *M. lepromatosis* infection in ERS as intended, as ERS on AR may not be infected with either leprosy bacillus at all. If infected ERS are present on AR, changes caused by the bacilli are likely to be minor, and without a reference point informing about the time since initial infection cannot be compared to results of clinically diseased ERS on BI.

Ectoparasites

Small mammals are regular hosts to a wide range of arthropod species (Maaz *et al.*, 2018). While ectoparasites were present on a large proportion of the ERS included in the study, they did not appear to have an immediate negative impact on their health. The observed patterns in parasite distribution on the host body could be explained by the ease with which an animal would be able to remove the parasite or could reflect preferred bite sites.

Parasite burdens are influenced by factors intrinsic to the host, such as age and immune status, and by environmental factors such as temperature or host and parasite density (Cardon *et al.*, 2011). Most ERS included in this study were adults, and, unfortunately, more detailed ageing in this group is not currently possible in live animals (Bosch and Lurz, 2012). As the immune status of a host cannot be determined just by external observation, the impact of host associated factors on parasite burdens cannot be assessed in this study.

However, information on temperatures and host densities are available for both locations and could explain the higher parasite burdens and more frequent occurrence of ticks along

with fleas seen on BI compared to AR. ERS densities are lower on AR, as is the ambient temperature throughout the year. Particularly during our early spring sampling sessions, the lower average temperatures may be linked to reduced ectoparasite activity. This is especially true for ticks, which are only occasionally active when temperature drop below 10°C (Perret *et al.*, 2000), something that is more likely to occur on AR at the times of our sampling than on BI. In spring 2018 it had in fact still been snowing on both islands shortly before our assessments commenced, which is most likely the reason why the lowest ectoparasite intensities were observed in this session.

Flea activity on small rodents has been shown to peak in April/May in a study carried out near Berlin (52° 31′ N, 13° 24′ O, average spring/autumn temperature 14°C, i.e. similar to BI), while tick larvae peaked in April and August and nymphs in June and July (Maaz *et al.*, 2018). It is thus likely, that the ectoparasite burdens observed in this study reflect the lower end of the spectrum of ectoparasite intensity the ERS in these populations may be facing throughout the year. The higher parasite burdens in the autumn sessions can be readily explained with the higher ambient temperatures, as can the more frequent observation of ticks.

The fact that no harvest mites were observed on AR could also be linked to environmental factors, however, further research would be necessary to determine whether harvest mites never occur on ERS on AR, or were only absent at the time of our sampling.

2.4.3. Carcass collection and post mortem assessment

Fewer animals were found dead on BI than on AR and more young animals were found. The lower total number could be associated with the smaller overall population or the absence of major roads or significant vehicle traffic on BI. On both Islands animals were mainly found by chance when people moved across the island. Therefore, where carcasses are concerned, the differences in how humans looking for ERS carcasses move around the islands is likely to have influenced our sample sets, and this bias needs to be considered when comparing data from the two locations.

Age, sex and reproductive status

Age distribution within the carcasses collected from both islands is almost certainly influenced by the location were carcasses where found, i.e. on the road (AR) vs. in the forest (BI). Juvenile animals are unlikely to venture onto roads and it can thus be explained that none of these young animals were collected from AR. The average crown-rump length of adult carcasses was similar on both islands (172.8mm (BI) vs. 172.6mm (AR)). As this sample set is smaller than that of live animals assessed this should not overrule the notion that ERS on AR may be slightly larger than on BI. The sex ratio for both islands was very balanced, while reproductive status is likely to be linked to the time of the year at which an ERS died, information which is not available for all ERS included. While the carcasses are

unlikely to be fully representative of the wider population, they allow an interesting and relevant snapshot into the population.

Body condition and weight

Observations made during post mortem assessments of ERS throughout this project have important implications for how a thin body condition should be judged. In several thin ERS carcasses good internal fat cover was observed. Therefore, thin body condition might not necessarily mean reduced fitness, but can occur in well fed ERS. Particularly on BI carcasses were in poorer body condition than animals assessed alive. This is likely due to the fact that these animals mostly succumbed to conditions that will have had a negative impact on their fitness and body condition prior to their death.

Generally, accidental and predation deaths as they dominate on AR are more likely to provide an insight into whether members of an ERS population are suffering from food shortages, than deaths that are the result of individuals succumbing to ill health, as appears to be more likely on BI.

General health status

The most frequently reported single causes of ERS deaths in Scotland are road traffic accidents (42.9%), trauma (11%), and starvation (9.8%), while circa 30% of the deaths were likely to be pathogen related (LaRose *et al.*, 2010). Other studies in Great Britain also identified road traffic as the main cause of ERS deaths (41.7% of deaths on the Isle of Wight, 50.7% on Jersey, 48% on Anglesey) in surveillance efforts relying on opportunistic sampling. Infectious disease was implied in up to 35% of the deaths recorded in these other locations (Simpson *et al.*, 2013; Shuttleworth *et al.*, 2015; Blackett *et al.*, 2018).

Looking at the carcasses collected on both islands, infectious disease was suspected to be at least a confounding factor in several animals on BI, but on AR the vast majority of the carcasses were healthy or coping with chronic conditions and killed by cars or predators. Therefore, findings on both islands seem to fit with the observations made in the extensive post mortem studies mentioned above: Where road traffic is present (AR), it is a major cause of squirrel deaths.

Ectoparasites

It is likely that more ectoparasites were present when the animals were still alive, but that they fell off the carcass post mortem or were brushed off when the carcasses were collected and frozen. The ectoparasite burdens observed on carcasses in this study are thus unlikely to allow extrapolation to the situation in the live population.

Conclusion

In summary, trapping and anaesthetic protocols used in this study were highly successful and provide an insight into a reasonably sized subpopulation of ERS on both islands. Two healthy ERS populations were picked for this study. In many respects they can be compared directly and factors limiting this comparability, i.e. habitat and climate differences, origin of populations, average size of ERS and likely causes of death based on location, were identified in this chapter. The two populations are suitable for studying the effects of leprosy on ERS as aspired, though where clinical leprosy is concerned, this will be limited to disease caused by *M. leprae*.

Chapter 3: Diagnosing leprosy in live ERS

3.1. Introduction

Diagnosing leprosy in any host is not a straightforward task. No diagnostic tool today is able to identify all individuals colonised by or infected with *M. leprae* or *M. lepromatosis* (Truman and Fine, 2010; Geluk, 2018). The effects of an infection with leprosy bacilli are largely determined by the individual host's cellular and humoral immunity (Bobosha *et al.*, 2014). Where large numbers of bacteria are present (MB cases, ca. 59% of annually reported human cases) the diagnosis is more readily confirmed than in PB cases (ca. 41% of annually reported human cases (WHO, 2016b)). The development of new diagnostic tests for leprosy, especially ones able to detect PB cases and colonised individuals at risk of becoming infected and/or diseased, is an active area of research (Lastória and de Abreu, 2014a; Sousa Lima *et al.*, 2019). For each host different tools have proven more or less useful and are usually combined to identify cases.

Leprosy in ERS was initially identified using histological and molecular methods (Meredith *et al.*, 2014; Simpson *et al.*, 2015). αPGL-I detection in body cavity fluids was added as a method in later studies (Avanzi *et al.*, 2016). In a small pilot which re-tested the original serum samples screened in the study presented by Avanzi *et al.* (2016), collaborating researchers at the Leiden University Medical Centre showed that their more recently developed UCP-LFA (van Hooij *et al.*, 2017) was able to detect αPGL-I in even more of those samples, thus indicating at least comparable, but most likely better, performance (Geluk and van Hooij, unpublished data).

Later post mortem screening efforts for leprosy in squirrels have relied primarily on PCR testing. This allowed the analysis of more samples in a shorter period of time than histological assessments, and is especially useful where no clinical lesions are present to guide the choice of location for histological sampling and specific experience in analysing tissue sections of leprous squirrels is limited (Butler *et al.*, 2017; Schilling, Avanzi, *et al.*, 2019). Overall, leprosy diagnosis in ERS has relied on being able to collect whole organs or at least large tissue sections (> 200mg) for histological assessment, DNA extraction and PCR, and on the presence of consistent clinical signs to guide sampling efforts.

This approach is possible and effective where squirrel carcasses are concerned. However, opportunistic sampling can introduce undesirable bias (Sikes and Gannon, 2011). In the case of ERS only animals would be included that died in locations where they can be quickly found and accessed by humans, for example on the ground near roads, feeders or popular woodland trails. These areas may not be frequented by all individuals in a population to the same extent, but may particularly attract sick, bold or habituated individuals. These may be at a different risk of infection than other members of the population and therefore data from these carcasses may over- or underestimate the presence of disease in the population.

Collecting tissue sections as large as those taken for diagnostics in carcasses is not an option in live ERS that must be released back to the wild immediately after sampling. Diagnostic methods do thus need to be adapted for use in live ERS, if less or differently biased sampling is to be attempted to get a fuller picture, for example of the prevalence of leprosy in an ERS population. Another scenario in which the ability to screen for leprosy in live ERS is particularly important is in the preparation of translocations. Such animal movements to reintroduce or reinforce ERS populations are ongoing (Shuttleworth, Lurz and Halliwell, 2015). Animals should be screened for diseases that could pose a threat to their own species, other species they will be sharing an ecosystem with, or to public health prior to being moved in such a program (Woodford, 2000), without impairing the individual's fitness for release and survival.

Ideally, diagnostic methods in live animals would reliably identify leprosy cases, be repeatable over time to allow for the comparison of leprosy status of an ERS and be minimally invasive, so that the ERS can be released back into the wild immediately after diagnostic samples have been collected.

In this part of the project clinical assessment, molecular and serological methods were adapted for use in live ERS under general anaesthesia. The hypothesis guiding this exploration was that diagnostic methods used in other host species and in deceased ERS can be successfully adapted to diagnose leprosy in live squirrels under field conditions.

The least invasive method is a visual clinical assessment. A wide range of people, from members of the public to wildlife rehabilitation and conservation professionals and veterinarians may observe ERS with clinical signs of leprosy in the UK. A standardised system is proposed to categorise such observations. This should allow individuals to follow changes over time and hopefully enable immediate recommendations for individual ERS management.

While leprosy lesions initially appear to be fairly pathognomonic (Avanzi *et al.*, 2016), there are other diseases that may have a similar appearance. Based on what is known from other host species the uniformity of clinical lesions observed in ERS during post mortem assessment appears unusual. The most visually similar disease is atypical histiocytosis (Smith *et al.*, 2017). Cancerous skin tumours could also resemble leprosy lesions. Other, life threatening, skin diseases that could be mistaken for leprosy in unusual cases or by an inexperienced observer include fatal exudative dermatitis (FED) caused by *Staphylococcus aureus* ST49 with *luk M* gene (Blackett *et al.*, 2018) and squirrel poxvirus (SQPV) infection (McInnes *et al.*, 2009; Everest *et al.*, 2017). It is therefore necessary to have further diagnostic techniques available when suspicious skin lesions are observed in an ERS.

It was assessed whether a small tissue sample that allows immediate release of the sampled ERS would suffice to extract DNA and establish the presence of *M. leprae*/*M. lepromatosis*

DNA using the PCR protocols already validated for ERS tissue sections. Ideally, molecular diagnostics for leprosy would fulfil two purposes in live ERS:

1. Allow the identification of non-clinically affected ERS that harbour the bacteria in their tissues without impairing the animals continued welfare, and
2. Allow the confirmation of a leprosy case where characteristic skin lesions are seen.

Lastly, the role that leprosy specific αPGL-I diagnostic tests can play in identifying cases of ERS leprosy from serum and blood samples was explored.

The results and experience with these tools throughout the project were then combined to propose a diagnostic decision tree and terms to describe ERS with different diagnostic outcomes. These could be used in any future studies into ERS leprosy and facilitate comparability between different studies, increase the opportunity for data sharing and re-use, as well as provide guidance as to which tool combination could be used in surveillance efforts and pre-release screenings.

3.2. Methods

3.2.1. Clinical assessment of leprosy lesions

Defining clinical leprosy lesions

The descriptions of ERS leprosy by Meredith *et al.* (2014), Simpson *et al.* (2015) and Avanzi *et al.* (2016) formed the basis for defining a clinical leprosy lesion in ERS. Thus, “bilateral areas of alopecia and cutaneous swelling of the snout area, lips, eyelids, pinnae and the distal aspect of the limbs” (Meredith *et al.*, 2014) or “crusty thickening of the pinnae, sometimes with keratinised or wart-like protuberances” (Simpson *et al.*, 2015) were expected. However, milder cases and different presentations could be present in live ERS. Therefore, the initial live assessment session in autumn 2016 was used to establish what would be considered a clinical leprosy lesion for this project. All ERS trapped in this session were assessed under general anaesthesia together with Professor Anna Meredith, an expert on ERS leprosy.

Categorising clinical leprosy lesions in ERS

Establishing a categorisation system for leprosy lesions in ERS was a multi-step process, each step aiming to improve the system in terms of meaningfulness, comparability of results and ease of application.

Step 1: Autumn 2016 – Establishing a subjective categorisation system

Initially, only the presence or absence and number of leprosy lesions was assessed visually. A range of images was taken from these individuals. Lesions were documented in prepared schematic drawings of squirrels, a black pen indicating healed traumatic changes unlikely to be related to leprosy, blue colour indicating lesions described in the context of leprosy with

intact skin, and red for ulcerating lesions indicative of leprosy or fresh traumatic wounds. Where necessary for clarity, a short description was added next to the drawing (Figure 24).

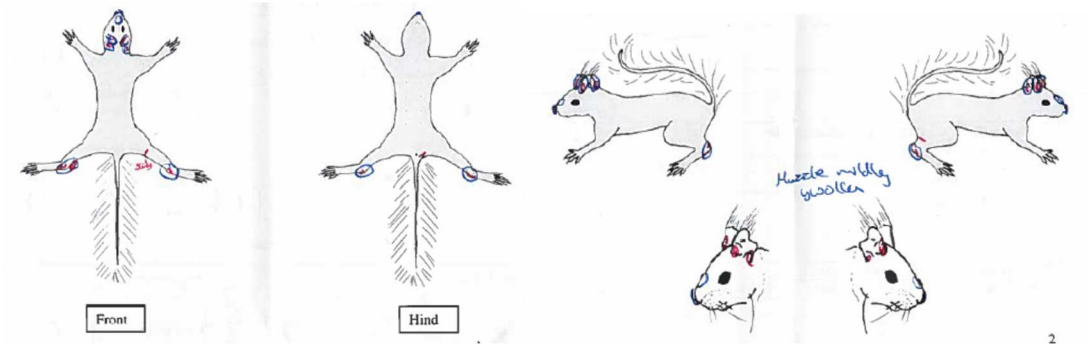


FIGURE 24: DOCUMENTATION OF LEPROSY LESIONS DURING ASSESSMENT

At the end of the session, subjective categories from 1 to 6 were assigned to each ERS based on the observations made during the assessment.

Step 2: Spring 2017 – Application and review of the subjective classification system

This 'subjective' classification system was applied to all ERS seen in spring 2017 on BI and AR and weaknesses and limitations of the system identified. Particular attention was paid to see if classification was consistent if used by the same investigator on separate occasions or when two separate individuals classified the same ERS. Another important aspect to assess was whether the system was suitable to document changes in lesions over time.

Step 3: Autumn 2017 – Establishing an objective classification system

Objective parameters to describe lesions (size, shape, presence and state of ulcerations) were trialled and then used throughout the session. A point score system was developed assigning values to different states of each parameter. It was decided to score the ERS body in body segments, to include the appearance of lesions in several body areas as a score increasing factor. This additive score was then used to establish a leprosy category for each ERS. At the same time, special attention was paid to the apparent well-being of the ERS seen with leprosy lesion, by now in some cases multiple times. The apparent welfare status was then linked to the categories the ERS had been placed in. Once the system was established, ERS seen in previous sessions were re-assessed and re-classified based on the notes and images available from them. This created comparable datasets and evaluated whether the objective system was able to illustrate changes in lesions that had occurred over time.

Step 4: Comparison of subjective and objective categorisation system

Ideally, the subjective and objective categorisation of leprosy lesions should correspond as this would allow the continued use of the subjective system (albeit observing the category names established in the objective system) in instances where full, detailed assessment of

all body areas and lesions is not possible in future studies. Table 10 details how the categories were expected to correspond.

TABLE 10: OBJECTIVE SEVERITY CATEGORIES AND HOW THEY SHOULD CORRESPOND TO THE SUBJECTIVE SYSTEM

Objective severity category	Would have been most likely categorised as … in the subjective system
1	2
2	3
3	4
4	4 or 5

Using R, the Spearman's rank correlation coefficient was calculated to assess whether the categories assigned to an ERS using the old 'subjective' categorisation system and the new 'objective' system were significantly correlated.

3.2.2. Molecular diagnostics in live ERS

Tissue sampling in live ERS

In previous studies a range of tissues (pinna, muzzle, spleen, liver, front- and hindlimb, and genitalia) from carcasses had been used to isolate and amplify *M. leprae* and *M. lepromatosis* DNA using PCR. Of these the pinna can be sampled in a live animal with ease.

Trying to balance minimal impact on the animal and sufficient sample size, it was decided to use a 2mm thumb style ear punch (World Precision Instruments Limited®), widely employed for marking mice in laboratory settings, for tissue sampling. Injuries of a similar size do occur in wild ERS and do not seem to reduce their ability to thrive. Unfortunately, we did not have previously diagnosed carcasses available to establish the tissue sample size needed for molecular analysis at the beginning of the project, and so the biopsy punch size was empirically chosen.

Sampling was done in spring 2017. It was decided to only sample animals without clinical signs of leprosy and no other changes to the ear that might put them at risk of developing leprosy lesions shortly (thinning of the coat, scaly appearance) to avoid potentially altering the onset or progression of lesions while the study was ongoing.

Two biopsies were taken from the left ear with the ear punch. The punch sites were chosen so as to avoid vessels near the rim of the ear that might increase the risk of continued bleeding and to avoid the remaining tissue around the punch site ripping to the rim of the ear once the animal was back in the wild. Punches were therefore taken caudal of a vertical axis through the pinna, at least 2 mm from the rim of the ear and 2-4 mm apart (Figure 25). Punch sites were prepared by clipping the fur and cleaning with chlorhexidine solution (Hibiscrub®). If any bleeding occurred after the punch was taken, pressure was applied with a clean compress until the bleeding ceased. If bleeding could not be readily stopped by

pressure alone, a small drop of tissue glue (GLUture Topical Tissue Adhesive (Zoetis UK)) was applied to the rim of the punch site.

FIGURE 25: PUNCH SITES FOR EAR TISSUE COLLECTION: BLUE DOTS MARK WHERE PUNCHES WERE TAKEN

The tissue samples were immediately placed in 70% ethanol and kept at +4°C during transport and storage until analysis. 70% ethanol inactivates both leprosy bacilli species. The metal punch was decontaminated in 70% ethanol/15% isopropanol for a minimum of 20 minutes before being air dried on a single use paper tissue to be ready for re-use.

Deoxyribonucleic acid (DNA) extraction

DNA extraction was performed at Moredun Research Institute in a containment level 2 facility. DNeasy® Blood and Tissue kits (Co. Qiagen) were used to extract DNA from the samples. The same method was applied to all tissue samples in this study. Where relevant adaptions were made, they are mentioned.

The punched-out tissue was removed from the ethanol, placed in a sterile petri dish and the alcohol was allowed to evaporate. Each tissue sample was transferred into a lysing matrix tube (Lysing Matrix B, MP Biomedicals) prepared with 0.1mm silica beads and 320µl ATL tissue lysing buffer (Qiagen DNeasy® Blood and Tissue Kit). For each batch of DNA extractions three extraction controls were included in the PCR used to screen for *M. leprae* and *M. lepromatosis* specific sequences: a tissue previously tested positive for leprosy bacilli DNA, a tissue known to be free from leprosy bacilli DNA and sterile distilled water (50µl). Unfortunately, there was not enough *M. lepromatosis* positive squirrel tissue available to run this control with every batch of DNA extractions. After one hour at room temperature the tissue was lysed in a FastPrepTM FP120 (Co. Thermo Savant) or a FastPrep®-24 Classic Instrument (Co. MP Biomedicals) for three cycles of 20s at 6m/s. Between cycles the samples were cooled on ice for five minutes.

Samples were then centrifuged in a microfuge at 15500g for five minutes to pellet the beads and debris. The supernatant (180µl) was transferred to a fresh microcentrifuge tube and proteinase K was added (20µl, activity 600mAU/ml solution, Qiagen DNeasy® Blood and Tissue Kit). After thorough vortexing the samples were incubated overnight at 56°C.

For DNA extraction the steps of the spin column protocol were followed as detailed in the Qiagen DNeasy® Blood and Tissue Kit handbook. Due to the small sample sizes a single step elution in 50µl AE buffer (Qiagen DNeasy® Blood and Tissue Kit) was performed as advised in the handbook. The eluted DNA samples were stored at +4°C until the PCRs were completed. They were then stored at -20°C.

PCR analysis

Specific primer pairs were used to perform PCR amplification as described by Avanzi *et al.* (2016); LPM244 (Singh *et al.*, 2015), amplifying a 244-bp fragment of the *hemN* gene of *M. lepromatosis*, and RLEP 7 and 8 (Monot *et al.*, 2009), amplifying a 500bp fragment of the RLEP repetitive sequence of *M. leprae*. It was decided to rely on the already established qualitative PCR protocol for this study instead of adding the additional work and time necessary to establish a new, quantitative protocol. A 50µl reaction volume containing nuclease-free water, GoTaq® Flexi buffer (Promega®), PCR Nucleotide Mix (Roche®), GoTaq® G2 Flexi DNA Polymerase (5u/µl), final concentration of each primer of 0.2µM and 3µl of DNA or control was used for PCR amplification. DNA from previous experiments was used as positive control (*M. lepromatosis* from previously confirmed squirrel case R30/13 and *M. leprae* from previously confirmed squirrel cases BR10/15, BR25/15, later BIC002/16) and distilled water as negative PCR control.

Amplification was performed using a BiometraTOne (Co. AnalytikJena AG). Samples were first denaturated at 95°C for five minutes followed by 40 cycles of 30 second denaturation at 95°C, 45 second primer annealing at 58°C, and 30 second extension at 72°C. Ten minutes were allowed for a final extension at 72°C.

Amplicon analysis was performed by electrophoresis using a 1.5% agarose gel, stained with GelRed (Biotium) in 0.5x TBE buffer, at 100 volts for one hour. 3µl of a 100bp DNA ladder (Promega®), which is to determine the size of double stranded DNA from 100-1,000 base pairs, was loaded before and after the controls and samples. A volume of 10µl (10-15mg tissue samples ‘full size sample’) or 15µl (2 mm tissue punches) of each sample was loaded. The amplicons were visualised by using an Alphaimager 2200 (Alpha Innotech) or a G:BOX F3 gel doc system and GeneSys software (version 1.6.1.0) (both Syngene). The same PCR protocol was applied to all DNA extracts in this study.

Assessing the impact of punch sampling on ERS

In the autumn 2017 and spring 2018 sampling sessions, punch samples were again collected only from animals without clinical leprosy lesions, to avoid altering their progress. To avoid “oversampling” of individual ERS, i.e. removing two 2mm more than twice throughout the duration of the study, returning ERS in these sessions were also not sampled. In autumn 2018, the final sampling session for this project, tissue punches were

taken from all ERS (see also “Ability to confirm leprosy cases using punch biopsies” below, p. 75).

It was noted whether the punch sites showed signs of acute or chronic inflammation, and whether in hindsight the punch sites were well positioned (i.e. as shown in Figure 25) or if one or both of them were slightly closer to the ear rim than aimed for. It was also noted whether the punch sites had not ripped at all, only showed minor ripping, or showed a major rip all the way to the rim of the ear, and which of the returning ERS had developed clinical leprosy lesions.

Statistical analysis

Ripping and position

The first null-hypothesis tested for the punch sampling sites was “Ripping of the sampling site is not associated with position of the punch”. A Pearson’s chi-squared test was carried out in R.

Ripping and time

The second null-hypothesis under investigation was “Ripping is not associated with the time passed since the original punch was placed”. A Pearson’s chi-squared test was carried out in R.

Ripping and non-iatrogenic injury

The third null-hypothesis tested was “The proportion of animals experiencing major rips after being punch sampled is equal to the proportion of animals experiencing non-iatrogenic tissue loss to their ears”. For this the presence of loss of ear tissue in animals that either were not punch sampled or did not return after being punch sampled (i.e. state of the ears was assessed only prior to punch sampling) and the presence of major rips by the time of the final assessment of the 30 returning ear punch sampled squirrels were compared. A Pearson’s chi-squared test was carried out in R.

Punch sampling and clinical leprosy lesions

Lastly, returning squirrels on BI were used to assess whether animals from which punch samples were taken were more likely to develop leprosy lesions at a later time point compared to those from which no punch samples had been collected. A Fisher’s exact test was carried out in R to address the hypothesis that the incidence of leprosy lesions is equal in squirrels that have and have not been punch sampled.

Ability to confirm leprosy cases using punch biopsies

In the final field session in autumn 2018, when monitoring ended, punch biopsies were collected from all ERS seen, regardless of their clinical status and whether samples had been collected previously. For ERS whose left ear had been previously sampled, the right ear was sampled now. Lesions were usually thicker than the biopsy punch could open, and,

there is a risk that punch holes in lesions would heal differently from those in apparently healthy tissues. As high bacterial loads could be reasonably expected in clinically diseased ERS based on carcass data (Avanzi *et al.*, 2016), it was decided to use an apparently healthy part of the ear to take the punch biopsies in these ERS.

3.2.3. Serological testing of ERS

Sample types

Three sample types were assessed: serum and blood drop samples for live animals and body cavity fluid for carcasses. They were analysed in conjunction with clinical and molecular information available for these ERS.

Serum samples

Blood samples were collected from the femoral vein under general anaesthesia in the field sessions autumn 2016, spring and autumn 2017 and spring 2018. The fur above the vein was clipped and the skin prepared using a chlorhexidine solution (Hibiscrub®). Consecutive samples from the same animal were assessed separately for this research segment. Serum was prepared at room temperature by centrifugation (10 minutes/2000g) and either diluted in buffer (1/50, 3µl in 147µl, see below, p. 77) and applied to the LFA strips immediately (spring 2018) or stored at -20°C until required (autumn 2016, spring and autumn 2017).

Blood drop samples and prick trial

Blood drop samples were obtained either using remaining blood in the syringe after ejection of the whole blood sample (autumn 2017) or via a skin prick using disposable 20µL Minivette® collection tubes (Heparin coated; Sarstedt) (spring 2018).

Prick sampling was attempted from the ear, the front footpad, the hind footpad or the tail respectively using a Sterilance Lite II (26 G x 3/32”; VITREX Medical A/S) or, more successfully, a sterile 25G x 5/8” needle. The prick site was prepared by clipping the hair and preparing the skin with chlorhexidine solution (Hibiscrub®) or ethanol. Sites that did not bleed at all and did not promise more success in additional attempts were not trialled further. Where the blood flow from the prick site was insufficient to fill the minivette or it was decided not to prick to reduce the time under general anaesthesia (n= 3), the minivette was filled from the whole blood sample.

Body cavity fluid (BCF)

Body cavity fluid (BCF) was collected from the thorax with a single use 2ml pipette during post mortem examination from 24 squirrels (20 adult, 1 subadult, 2 juveniles, 1 age unknown). From four of these serum samples taken ante-mortem were available as well. One of the squirrels in this subset died from sudden cardiac arrest during the anaesthesia, while three were humanely euthanised in accordance with the home office licence

conditions. The other 20 carcasses had been found dead in the wild. All carcasses were stored at -20°C until the post mortem examination was carried out.

αPGL-I up-converting phosphor (UCP) lateral flow assay (LFA)

A quantitative αPGL-I UCP LFA, well established for leprosy serodiagnosis in humans (A. van Hooij *et al.*, 2016), along with the necessary buffers was provided by collaborators Annemieke Geluk and Anouk van Hooij at Leiden University Medical Centre. As a Packard FluoroCount microtiter plate reader adapted for measurement of the UCP label (980nm IR excitation, 550nm emission) is needed to read the assay, the test readings were done by Anouk in Leiden.

All samples were diluted 50-fold in high salt lateral flow buffer (HSLF; 100 mM HEPES pH 7.5, 270 mM NaCl, 1% (w/v) BSA, 0.5% (v/v) Tween-20, later batches additionally contained Triton X-100 to lyse red blood cells) provided in lyophilised form by our Dutch collaborators. In the field, the HSLF buffer was dissolved in still, commercially available bottled water at the start of each sampling session as instructed by our collaborators and stored at +4°C for the three sampling days. Body cavity fluid samples were stored frozen and then thawed prior to dilution with HSLF buffer. All samples collected in 2016 and 2017 were shipped to the Netherlands and processed by Anouk van Hooij and I at Leiden University Medical Centre (LUMC), Netherlands, in January 2018. For samples collected in spring 2018, the UCP-LFA sticks were taken to the field and the αPGL-I UCP LFA ran here at the end of each sampling day (as described below). The dried test sticks were sent to the Netherlands for reading, along with four diluted serum samples, for which LFA sticks had not been available.

Of each sample/buffer mix 50µl were added to a well of a 96-well microplate (Greiner Bio-One). UCP-LFA strips were labelled on the back with the sample number and placed in the sample well with the UCP-containing sampling pad in the buffer. The strips were left in the well until all liquid was absorbed and the strip had dried completely. They were stored attached to a piece of paper with tape across the absorbent pad and read in the FluroCount™ at LUMC. Results were provided as the ratio value between test and flow-control signal based on relative fluorescence units measured at the respective lines.

Statistical analysis

For each sample three values were noted from the strip reading: the area under the curve of the sample signal (T), the area under the curve of the flow control signal (FC) and the Ratio (T/FC). A test was considered negative when T= 0 or T/FC is below the threshold for positivity. The threshold for positivity was initially calculated in Leiden from the sample sets taken from autumn 2016 till autumn 2017 and later refined when the samples from spring 2018 were added using Youden's index (Fluss, Faraggi and Reiser, 2005). I was involved in preparing all data for analysis, but statistical analysis for these experiments was carried out by Anouk van Hooij as part of our collaboration.

Graphpad Prism version 7.00 for Windows (GraphPad Software, San Diego CA, USA) was used to perform Mann-Whitney U tests, Kruskal-Wallis test with Dunn's correction for multiple testing and a Pearson correlation test.

3.3. Results

3.3.1. Clinical assessment of leprosy lesions

Defining clinical leprosy lesions

Out of the 26 animals seen in autumn 2016, 18 squirrels were deemed free of clinical leprosy lesions and eight were classed as showing clinical signs of leprosy. For one of these ERS serology results and observations in following sessions indicated that the chin lesion observed on this occasion was not a leprosy lesion. There were no other obvious lesions. Therefore, for the purpose of defining a leprosy lesion this animal was excluded. Figure 26 to Figure 32 inclusive show the clinical skin lesions seen in the remaining seven ERS in the order they were seen in the field.

FIGURE 26: SINGLE NODULAR LEPROSY LESION ON THE CAUDAL RIGHT EAR RIM OF ERS BI003_16

FIGURE 27: NODULAR, BALD, WELL DEFINED LEPROSY LESIONS ON THE LEFT EAR AND BOTH HOCKS OF ERS BI009_16

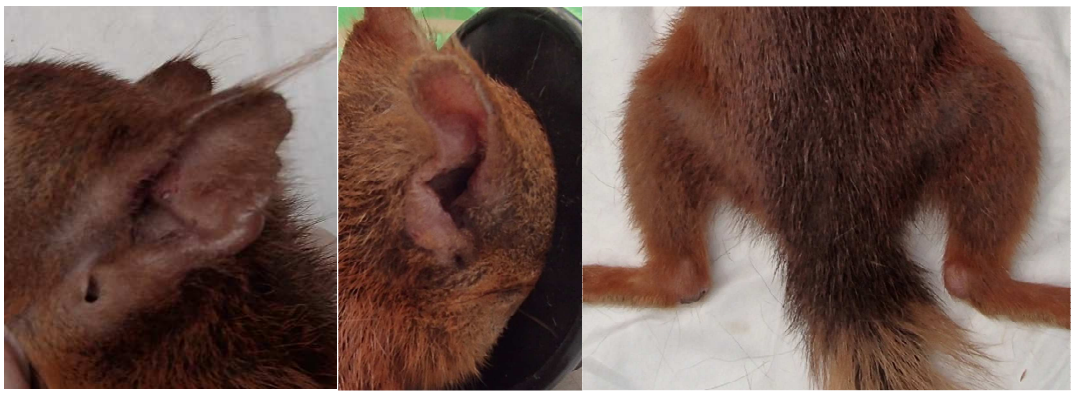


FIGURE 28: ELONGATED AREAS OF BALDING, THICKENED SKIN ON THE CAUDAL AND CRANIAL RIM OF BOTH EARS ALONG WITH NODULAR LESIONS ON BOTH HOCKS; ULCERATION ON THE LEFT HOCK (BI010_16)

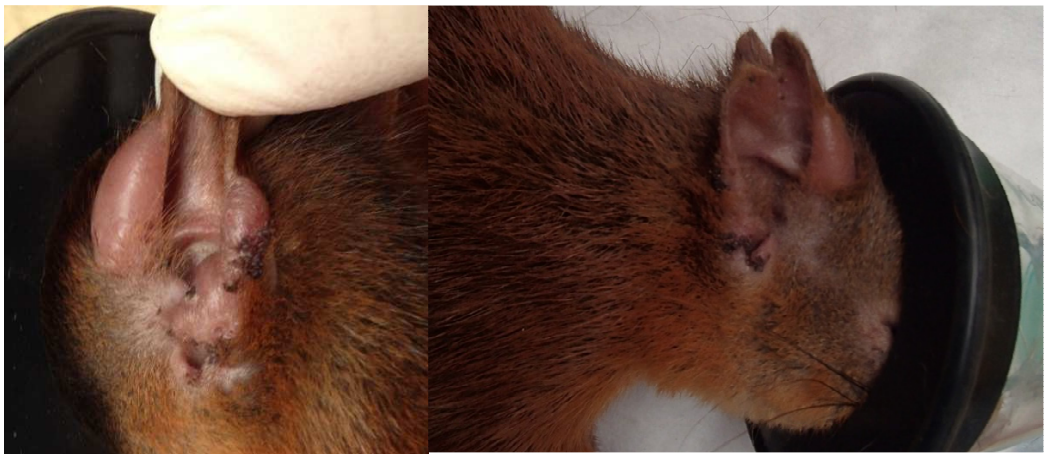


FIGURE 29: ELONGATED, BALD, SHINY SWELLINGS ON THE CRANIAL RIM OF BOTH EARS IN ERS BI016_16. LARGE NUMBER OF TICK LARVAE ATTACHED TO CAUDAL RIM OF LEFT EAR.

FIGURE 30: VERY SMALL BALDING, SHINY, SLIGHTLY BULBOUS SKIN PATCH ON THE OUTER CRANIAL RIM OF THE RIGHT EAR (BI017_16)

FIGURE 31: OBVIOUS BALD, SHINY, BULBOUS SWELLING ON THE INSIDE OF THE CRANIAL RIM OF THE LEFT EAR; THICKENING AND BALDING, ALONG WITH MILDLY CRUSTY APPEARANCE OF THE CAUDAL RIM OF THE RIGHT EAR (BI023_16)

FIGURE 32: SMALL BALD, BULBOUS SWELLING CRANIAL RIM OF THE LEFT EAR (BI025_16)

No lesions resembling the description by Simpson *et al.* (2015) were seen on BI. Based on these observations, leprosy lesions appear to form first on the ear rims and/or on the hocks. The balding and shininess of the skin of lesions was very consistent, as was the firm-rubbery consistency of the swellings.

Categorising clinical leprosy lesions in ERS

Step 1: Autumn 2016 – Establishing a subjective categorisation system

Based on the expertise acquired in this first field session and images of earlier cases and suspect cases available categories were defined to reflect the range of clinical signs that had been observed. This system is detailed in Table 11 and Figure 33. This categorisation system was applied retrospectively to animals seen in autumn 2016 and applied to squirrels seen in the spring 2017 field session.

TABLE 11: CATEGORISATION SYSTEM EMPLOYED IN AUTUMN 2016 (RETROSPECTIVE) AND SPRING 2017

Category	Description
0	No abnormalities indicating leprosy (with or without ear tufts depending on season)
1	Suspicious (Fur on ears thin, skin seems slightly shiny, muzzle might or might not be swollen)
2	Small single leprosy lesion (hairless, shiny swelling on one ear, and/or generalized swelling of the muzzle)
3	Small multiple leprosy lesions (hairless, shiny swelling on both ears or several on one ear, generalized swelling of the muzzle or hairless nodular swelling on the hind legs
4	Severe non-ulcerating lesions (cauliflower appearance of multiple shiny lesion on ears, muzzle and/or feet)
5	Severe ulcerating lesions on ears, muzzle, and or hock (same shiny, hairless lesions have ulcerated and might be affected by secondary infection)
6	Unusual lesions that could be leprosy associated (crusts on ears)

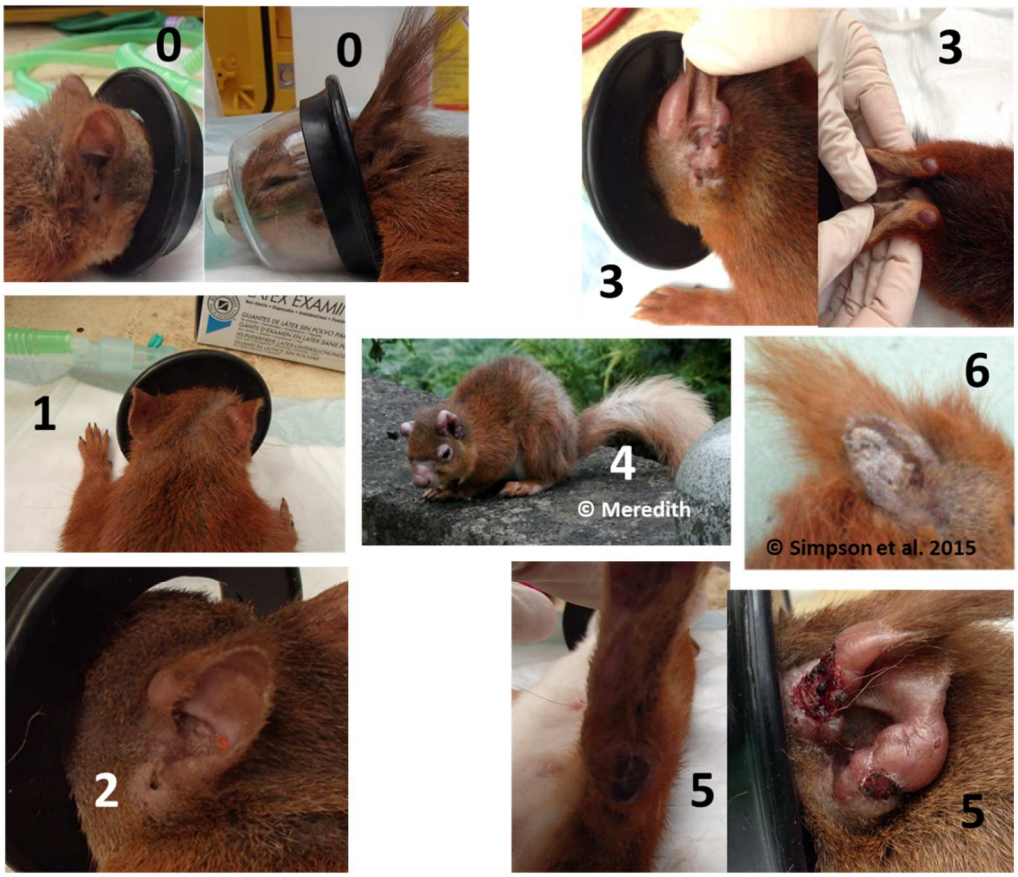


FIGURE 33: IMAGES USED TO DESCRIBE THE INITIAL CATEGORIES USED IN AUTUMN 2016 (RETROSPECTIVE) AND SPRING 2017

Step 2: Spring 2017 – Application and review of the subjective classification system

While the subjective classification was sufficient for identifying potential cases, it proved unable to reflect the intensity of changes in an individual's condition over time and thus insufficient for research purposes. It was also inherently sensitive to inter- and intra-investigator variation.

The numbers assigned to the different categories also proved slightly counterintuitive, as individuals classed as suspicious could not be confirmed through laboratory results. This category was therefore highly speculative and its usefulness appeared more and more questionable as the project progressed.

New findings also indicated that a ‘cauliflower’ appearance followed repeated ulceration and healing cycles, in which case it was inaccurate to assign a seemingly lower category to animals with cauliflower lesions than to those with currently ulcerated lesions.

Lastly, the categories were purely descriptive and not directly associated with the welfare impairment an ERS was likely to experience. It was thus not useful for making management action recommendations based on category.

Step 3: Autumn 2017 – Establishing an objective classification system

Only lesions of the previous categories 2 to 5 were treated as typical clinical leprosy lesions and were subjected to full categorisation. Individuals resembling the previously used categories 1 and 6 were noted and images taken, but they were not scored or categorized within the objective system for clinically leprosy positive ERS, as infection was not detected in these ERS.

To collect the information for the calculation of the numerical lesion score the ERS’s body was divided into six sections (1= left side of head, 2= right side of the head, 3= left upper body and forelimb, 4= right upper body and forelimb, 5= left lower body and hindlimb, 6= right lower body and hindlimb, Figure 34, p. 84). Where several lesioned areas occur in the same body sector, the score parameters were assigned based on the most intense lesion within the sector. The size of the lesions (<2mm, <5mm, <10mm, >10mm) was measured. To describe lesion shape four options were defined (A= one lesion with clearly defined rim, or area of balding skin; B= several lesions, separated with clearly defined rim; C= several lesions, merging, rim not always clear; D= cauliflower appearance due to merged lesions). The presence of ulcerations was noted as either “no”, “could be traumatic”, or “ulceration”. Where an ulceration was present it was further noted whether it was dry, bleeding or purulent.

Points were assigned following a pre-determined key (Table 12) for the findings in each body area and added per area and for the full body. This delivered the first outcome of the system, the full body score, which can reflect small changes in lesions that occur over time in detail. This is mainly relevant for research purposes.

TABLE 12: POINTS ASSIGNED PER BODY SECTION IN STEP 3 OF THE CATEGORISATION PROCESS (A= ONE LESION WITH CLEARLY DEFINED RIM OR JUST BALDING SKIN, B= SEVERAL LESIONS, SEPARATED WITH CLEARLY DEFINED RIM, C= SEVERAL LESIONS, MERGING, RIM NOT ALWAYS CLEAR, D= CAULIFLOWER APPEARANCE DUE TO MERGED LESIONS)

Points	0	1	2	3	4
Lesion size [mm]	None	<2	<5	<10	>10
Lesion description	None	A	B	C	D
Ulceration*	None	Trauma, not true ulcer	-	-	Yes
Ulcus description	None	-	Dry	Bleeding	Purulent

**Ulcerations are expected to have a high impact on welfare, points were thus not assigned continuously but in relation to the perceived welfare impact*

Based on the scores reached in the individual body sections and the number of affected body sections, the ERS was then assigned to one of four categories (1= no more than two body sections affected with no more than four points per section; 2= no more than three body sections affected, no more than six points per section; 3= up to four body sections affected, no more than eight points per section; 4= three or more body sections affected, scores of eight and more reached in at least one section). Note that a maximum score per individual section is defined above which an ERS is automatically placed in the next higher category, even if lesions are locally restricted or for some reason information is not available for all body sections. The scoring process is summarised in Figure 34. These severity categories are the second outcome of the scoring system.

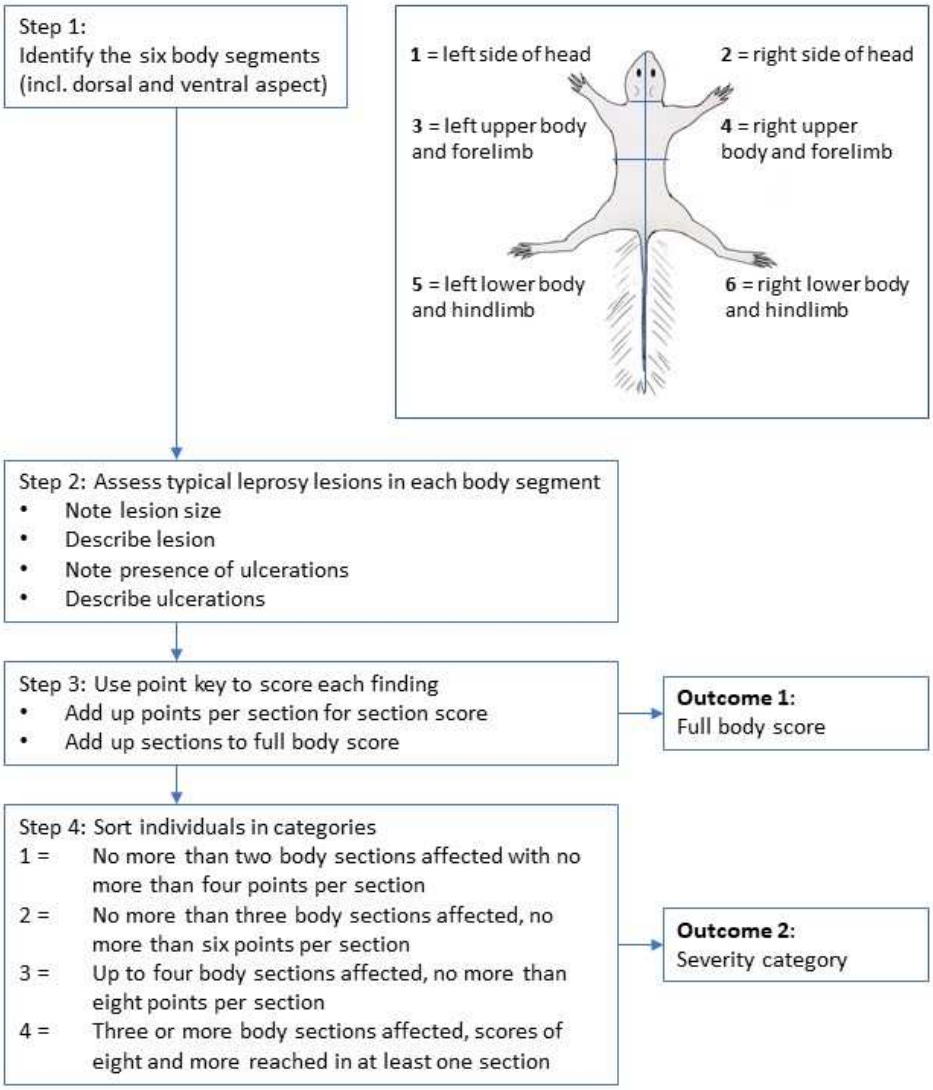


FIGURE 34: PROCESS OF CATEGORISING TYPICAL LEPROSY LESIONS

Based on veterinary expertise and experience gained throughout this project the severity categories were linked to a welfare state estimate and action recommendations (Table 13). It was possible to retrospectively apply this system to ERS seen in previous assessment sessions based on stored images and assessment sheets.

TABLE 13: SEVERITY CATEGORIES AND THEIR WELFARE IMPLICATIONS

Severity category	Description
1	Mild case, no welfare impairment expected, no immediate action
2	Mild to moderate case, no significant welfare impairment expected, no immediate action
3	Moderate case, welfare may be impaired, monitoring recommended
4	Severe case, welfare likely to be impaired, veterinary assessment of ERS recommended

Step 4: Comparison of subjective and objective categorisation system

In total, both systems were used in 69 assessment events. 26 squirrels trapped in autumn 2016 were only assessed based on our documentation and photographs. 43 were grouped according to the original system when seen in the field in spring 2017 (13 were returnees from the first trapping session) and then reassessed after the objective system had been conceived. Placement of animals in the different categories is indeed correlated well between the subjective and objective system (Table 14), with a Spearman's rank coefficient of ρ= 0.91.

TABLE 14: CORRELATION BETWEEN SUBJECTIVE AND OBJECTIVE CATEGORIES, P<2.2E-16, RHO=0.91

Old lesion Category	New lesion category				
	0	1	2	3	4
0	47	-	-	-	-
1	5	-	-	-	-
2	-	7	1	1	-
3	-	1	1	-	2
5	-	-	-	-	4

3.3.2. Molecular diagnostics in live ERS

Tissue sampling in live ERS

In total, 31 tissue punch samples were collected in spring 2017, 17 on AR and 14 on BI. *M. leprae* DNA was successfully isolated from six of them. All positive results came from BI. This showed that in principle the chosen sampling method and site were suitable to confirm the presence of *M. leprae* DNA in clinically healthy ERS.

Assessing the impact of punch sampling on ERS

Over the total course of this study 115 tissue punch samples were analysed. No leprosy bacilli DNA was present in 101 (87.8%). Fourteen (12.2%) samples contained *M. leprae* DNA (n_{BI}= 13, n_{AR}= 1). *M. lepromatosis* was not isolated from any of the samples.

Thirty punch sampled ERS returned in later assessment sessions. In none of them were signs of acute or chronic inflammation observed around the punch sites. Six animals returned twice, the other 24 once. Out of the 30 animals three developed leprosy lesions after 12 (n= 2) and 18 (n= 1) months, respectively.

Figure 35 shows fresh and healed punch sites. The changes to the top punch site in the middle image illustrate what was classed as minor ripping and a major rip can be seen in the top punch site on the right.

FIGURE 35: LEFT: ERS EAR IMMEDIATELY FOLLOWING TISSUE COLLECTION, ALSO SHOWING A NON-IATROGENIC INJURY. MIDDLE: 6 MONTHS AFTER SAMPLING, OPTIMAL HEALING ON THE VENTRAL PUNCH SITE, MINOR RIPPING ON THE CAUDAL RIM OF THE DORSAL PUNCH SITE. RIGHT: MAJOR RIP OF A PUNCH SITE TO THE RIM OF THE EAR.

Ripping and position

In 17 animals the punch sites were classed as well placed, in one the top punch site was as intended, while the bottom punch site was slightly closer to the rim than ideal. In the remaining 12 animals the punch sites were a bit closer to the rim than ideally aspired. At the time of the first re-assessment no rupture was seen in 20 animals (66.7%), minor rips were present in five animals (16.7%), and major rips were seen in five squirrels (16.6%). In three out of the six animals re-assessed twice, major rips occurred by the time of their second re-assessment. Leaving to the best of our knowledge 56.7% of the animals without rips and 26.6% with major rips. Major rips only occurred in one of the punch sites in all of the affected ERS. Only once was this the bottom punch site, in the other seven ERS the top punch site ripped. The hypothesis that placement of the punch site is not associated with ripping could not be rejected based on the data (Pearson's chi-squared test, n= 30, p=0.1815).

Ripping and time

Regardless of time passed since the punch biopsy was taken, most animals did not show ripping of their punch sites. Out of the 24 animals re-assessed once, 17 were seen six months after the punch had been taken, four were seen 12 months later and three were seen again after 18 months. Four animals re-assessed twice were seen six and 12 months post initial sampling, and two were seen after 12 and 18 months. However, some ERS were seen with major ripping at each time point, and ideally ways should be found to reduce the proportion of these animals (Figure 36). Ripping of punch sites is not significantly associated with the time that has passed since a punch sample was taken (Pearson's chi-squared test, n= 36, p=0.5841).

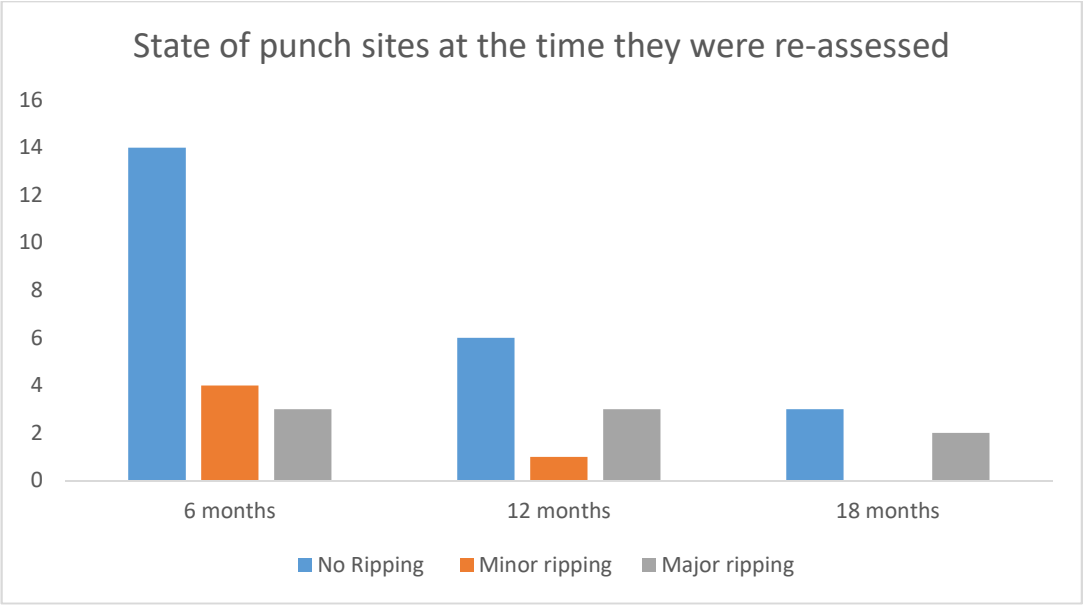


FIGURE 36: STATE OF PUNCH SITES OBSERVED IN 36 REASSESSMENTS OF 30 DIFFERENT ERS.

Ripping and non-iatrogenic injury

Out of the 152 included assessments (non-returning animals), non-iatrogenic loss of substance was seen in 27 squirrels (n= 12 on AR, n= 15 on BI), while, as mentioned before, eight animals out of the 30 reassessed punch sampled animals experienced major rips. The proportion of animals experiencing non-iatrogenic loss of substance to their ears throughout their lives is similar to that of punch sampled animals experiencing major rips (Pearson's chi-squared test, n= 182, p= 0.38).

Punch sampling and clinical leprosy lesions

A total of 31 animals were seen more than once on BI. Punch samples were collected from 21 returning individuals, of which three later developed leprosy lesions (14.3%). Out of the 10 returning animals from which no punch samples were collected, four already had leprosy lesions when first assessed and were excluded. Of the remaining six animals that did not show signs of leprosy in their first assessment, two developed leprosy lesions during the duration of this study (33.3%). Based on our limited sample size punch sampling did not result in an increased risk of developing leprosy lesions (Fisher's exact test, n= 27, p=0.3031, 95% CI= 0.028-5.482, OR= 0.35).

Ability to confirm leprosy cases using punch biopsies

In autumn 2018 punch samples were collected from 52 ERS in total. Six (all BI) showed clinical signs of leprosy, 46 did not (23 from BI, 23 from AR). *M. leprae* DNA was isolated from only three animals. Two clinically positive animals from BI, with severe lesions, and one clinically negative animal from AR. Two of the clinically positive ERS from which no *M. leprae* DNA was isolated had early, mild lesions (category 1, score 3 and 4, respectively). In one the lesion was on the hock, not the ear that was sampled, in the other the punch was

taken from the left ear, while the lesion was on the right. The other two ERS with clinical lesions were more severely affected (category 2, score 12, lesions on right ear and scrotum, punch taken from left ear; category 4, score 18, lesions on left ear and both hocks, punch taken from right ear).

3.3.3. Serological testing of ERS

αPGL-I

Serum samples

In total, 132 serum samples were available from 90 individual ERS (87 adult, 3 subadult) to validate the αPGL-I UCP LFA. Information on the presence of clinical lesions was available for all ERS assessments. Clinical lesions were present in 25 instances. Tissue samples screened for the presence of leprosy bacilli DNA were available for 64 individuals, who were clinically negative at the time of sampling. From 11 of these *M. leprae* DNA was isolated. For 25 clinically negative ERS no PCR result was available (reasons see p. 74).

ERS with clinical lesions had significantly higher αPGL-I ratios than those without lesions and a positive ($p<0.0001$, Kruskal-Wallis test with multiple Dunn' correction) or negative ($p<0.0001$) PCR result. ERS of unknown PCR status and without clinical lesions were excluded from this analysis. The αPGL-I levels of squirrels without clinical lesions did not differ significantly between those with a positive or negative PCR result ($p>0.9999$, Mann-Whitney U test). The level of αPGL-I is correlated with lesion severity reflected by the lesion score in ERS (Pearson correlation, $p<0.0001$; $R^2= 0.64$).

The αPGL-I UCP-LFA has a sensitivity of 88% and a specificity of 96% using a cut-off ratio of >0.1 to confirm infection with *M. leprae* in ERS with suspicious skin lesions. Graphs illustrating these results are presented in the joint publication "*Detection of humoral immunity to mycobacteria causing leprosy in Eurasian red squirrels (Sciurus vulgaris) using a quantitative rapid test"* (Schilling, Hooij, *et al.*, 2019) open access. The paper also details a blood drop and prick trial and the results from body cavity fluids.

Blood drop and prick trial

Blood drop samples were collected during 65 assessments, comprising 26 syringe samples and 39 minivette samples. For each a corresponding serum sample was available. Eight of these sample pairs came from squirrels with clinical leprosy lesions, and 57 from animals without clinical lesions. From five of these clinically negative ERS *M. leprae* DNA was isolated via PCR. The αPGL-I levels detected in serum and blood drops showed significant correlation (Pearson correlation, $p<0.0001$; $R^2= 0.9$).

The blood flow from the prick site was insufficient to fill the minivette in 32 attempts. Successful collection of enough blood to fill the minivette was only possible from the last third of the tail in 4 out of 25 attempts. Attempts to prick the ear, front and hind footpad were

abandoned after four, one and six attempts, respectively. The front footpad did not bleed at all following pricking. The four successful attempts on the tail were made after the hair of the prick site was clipped very short, the site was disinfected with ethanol, and the tail was warmed on a heat pad after the ethanol had evaporated and before the prick was attempted.

Body cavity fluid

BCF was collected from seven carcasses with leprosy lesions and detectable *M. leprae* DNA, for three of which ante-mortem serum samples were available as well. Additional BCF samples were available from three *M. leprae* PCR positive carcasses without leprosy lesions and 14 ERS carcasses clinically and PCR negative.

Correlation of αPGL-I levels and clinical signs was weaker in BCF than in samples obtained from live ERS. Only four of the seven clinically diseased ERS carcasses (57%) had αPGL-I levels above the cut-off ratio. This included the three animals for which serum was available as well. In these αPGL-I levels were lower in samples collected during post mortem examination, despite the ERS being frozen immediately after euthanasia and thus much better preserved than squirrel carcasses found in the wild usually are (Table 15).

TABLE 15: COMPARISON OF ALPHA-PGL-I IN SERUM AND BODY CAVITY FLUID OF THREE EUTHANISED ERS

Animal	BI010_16	BI052_17	BI062_17
Serum αPGL-I ratio	0.74	1.85	0.16
BCF αPGL-I ratio	0.29	0.28	0.11

3.4. Discussion

The leading hypothesis of this part of the study, that diagnostic methods used in other host species and in deceased ERS can be successfully adapted to diagnose leprosy in live ERS under field conditions, is supported by the results presented here.

It was shown that molecular methods can add information beyond that accessible in a clinical assessment, while serological methods can confirm the clinical diagnosis of leprosy in ERS. The latter is important when clinical signs are atypical and clinically not clearly distinguishable from other skin conditions that could occur in ERS. The diagnostic methods introduced here are a useful and suitable toolkit for leprosy diagnostics in live ERS.

3.4.1. Clinical assessment of leprosy lesions

Defining clinical leprosy lesions

The clinical presentation of leprosy lesions is more variable than previously published data from carcasses had suggested (Meredith *et al.*, 2014; Avanzi *et al.*, 2016). Based on the data presented in this chapter, clinical leprosy lesions will be defined as “skin areas of marked shininess and local hair loss, in which firm-rubbery swellings develop”. Especially, early lesions can be very discreet and difficult to identify.

More information on the clinical presentation of leprosy in ERS based on all data collected throughout this study can be found in chapter 4 (p. 109). It is still possible that the presentations observed on BI only represent part of the leprosy spectrum in ERS as they are different from the presentations observed on the Isle of Wight (Simpson *et al.*, 2015). However, the warts and crustiness observed there could also be the result of multifactorial problems.

Categorising clinical leprosy lesions in ERS

The desired outcomes of categorising leprosy cases in ERS are different from what is needed in humans. In people the main aim of identifying a leprosy patient is assigning the patient to a category to place him/her in an adequate treatment group quickly and prevent further transmission (WHO, 2016a). Further categorisation of clinical cases aims to assess the severity of existing leprosy related disability and how it changes during treatment (van Brakel, Reed and Reed, 1999), or reflect histological changes within the clinical lesions in detail for scientific purposes (Reibel, Cambau and Aubry, 2015). In wild ERS long term antibiotic treatment cannot be reasonably accomplished. Visual signs of disability that could imply a loss of nerve function, such as extensive injuries or loss of digits correlated with the occurrence of clinical signs of leprosy, were not observed in this study. In live ERS, tissue biopsies large enough for full histological assessment may make an immediate release of the ERS impossible, and thus collecting detailed information on lesion histology is limited to carcass assessments. In ERS the purpose of categorising clinical cases is to allow guidance on how to manage an animal that has been seen with lesions by, for example, members of the public or wildlife rangers. With such different goals, a clinical categorisation system for ERS necessarily had to be different from the systems established for humans, though it can borrow ideas from them.

The initial attempt to create a subjective system with descriptive categories, was based on the idea that the system should be easy to communicate and teach, preferably just using one typical picture per category. This would have allowed provision of an image catalogue to all those working with ERS in the wild and they would have easily been able to place whichever ERS they saw in one of the categories.

However, the subjective system proved prone to variable category placement of ERS by different investigators and even for the same investigator on different days, and it did not allow follow-up of lesion progression in sufficient detail for this particular research study. Subtle changes in lesions that occurred throughout the time of the study, required a more differentiated documentation of lesion state during the clinical assessment.

The objective system addressed these problems by introducing the full body score in addition to an overall lesion category. The detailed full body score will allow any investigator to follow leprosy lesion progression in ERS over time. The fact that the subjective and

objective system correlate well, means that the objective categories can still be illustrated with similar, easily communicated images. This, together with the option of using a high score in a single body area to immediately place an ERS in a high severity category could be very important. It may allow one to assign at least a tentative category to an ERS when only part of the body is available for assessment. This is for example the case when concerned members of the public take images of affected animals and ask for advice.

Based on observations made throughout this study, ERS in category 1 and 2 should be able to survive and thrive in the wild, but may be or become a source of infection to others. As transmission is not fully understood in any host at this point, it is very difficult to judge the role of individuals in spreading the disease. ERS in category 3 should be monitored where possible (for example if they return to a specific feeder) to pick up any worsening in their condition. They may be or become a source of infection for others. ERS in category 4 should be assessed by a veterinarian and humane euthanasia may be indicated. Some cases may be able to continue to thrive but may be or become a source of infection for others. Thus, the four severity categories (1= mild, 2= mild-moderate, 3= moderate, 4= severe) system developed here can be used both in hands-on and hands-off assessments of leprous ERS to guide management decisions. Clearly, assigning an ERS to a certain category based on a hands-on assessment, calculation of body scores and translation into a category will be less prone to miscategorisations than hands-off assessments and assessments of images that only show part of an ERS body.

3.4.2. Molecular diagnostics in live ERS

This study has shown that is possible to obtain sufficiently large tissue samples for molecular diagnostics from live ERS without compromising animal welfare.

Tissue sampling in live ERS

The successful isolation and detection of *M. leprae* DNA from 2mm tissue samples from the pinna implies, that the empirically chosen sampling site and type were suitable for molecular leprosy diagnostics in ERS. Molecular diagnostics are successfully used in tissue samples from other host species, such as humans or NBA (Martinez *et al.*, 2014; Sharma *et al.*, 2015). However, it had not been explored previously how small a sample may be. In humans skin biopsies are mostly taken from individuals displaying clinical symptoms of leprosy, and biopsy sites are chosen from active lesions whenever possible. Biopsies are collected in a size that also allows for histological assessment (Martinez *et al.*, 2014). Ear tissue samples taken from NBA are described as “ear fragments” or “ear notches” but no exact size is reported (Loughry *et al.*, 2009; Pedrini *et al.*, 2010). In this study it was shown that a 2mm tissue fragment from the ear of an ERS provides sufficient material for leprosy diagnostics, at least in this species.

Other sample types that have been explored in humans and NBA to avoid invasive tissue sampling for molecular diagnostics include blood, urine, nasal swabs, hair bulbs, and slit skin smears. They were however found to be less effective and/or harder to interpret that skin biopsy samples (Martinez *et al.*, 2014; Sousa Lima *et al*., 2019). In ERS such samples would incur additional problems due to the small size of the species, which restricts the blood volume that can be collected at one time to a millilitre. Swabs would require to be specially adapted to fit into the nares of an ERS without risking injury while still gaining sufficient sampling material for analysis. Slit skin smears would leave a wound that even when closed with tissue glue or stitches, could mean an ERS is not immediately fit for release following the sampling. Thus, the chosen sampling location and method appear to be a good option for molecular diagnostics in live ERS. Whether other sampling locations would hold the potential for increased diagnostic sensitivity is explored in more detail using carcass tissues in chapter 4 (p. 109).

Assessing the impact of punch sampling on ERS

A diagnostic method should never impair an animal's wellbeing more than absolutely necessary or reduce its ability to survive. Ripping of punch sites was a complication that was observed several times in this study. It was difficult to prevent even with careful placement of the punches. However, not all punch sites ripped, and some were intact up to 18 months after sampling. The severity of tissue loss seen where punch sites ripped was similar to that observed as a result of non-iatrogenic injuries the ERS experienced. Taking punch samples did not appear to increase the risk of developing leprosy lesions.

Taking tissue punch samples provided information on the presence of *M. leprae* DNA in clinically healthy ERS that could not be gathered in any other way. The impact of even ripped punch sites on the animal appears to be small and within the range of adversaries a member of this species may experience naturally. Sampling did not relate to an increased risk of developing leprosy lesions for the ERS. The value of the information gathered compared to the impact it is having on the ERS justifies the continued use of this method until better methods are developed. Additionally, sampling under general anaesthesia, eliminates acute pain perception during the sampling. From similar injuries that humans regularly inflict on themselves (piercings) it appears to be quite generally accepted that they do not cause any continued pain unless they become inflamed or infected, something that was not observed in ERS in this study.

Ability to confirm leprosy cases using punch biopsies

A limitation in the method discussed above is that it is not possible to determine if a negative PCR result indicates the true absence of bacteria or whether a bacterial load below the not currently defined threshold for detection could still be present. Observations in other host species show that some individuals are completely resistant to an infection with *M. leprae*

(Bennett, Parker and Robson, 2008; Balamayooran *et al.*, 2015). It is therefore possible that at least some of the PCR negative ERS identified in this study are truly free of leprosy bacilli.

Assessing the correlation between molecular and clinical leprosy diagnosis in ERS offered additional information on the matter. For human leprosy patients PCR sensitivity varies depending on where a patient is on the leprosy spectrum (Scollard *et al.*, 2006; Reibel, Cambau and Aubry, 2015). The reported sensitivity for the detection of PB cases varies between 30 and 83% (Reibel, Cambau and Aubry, 2015). Sensitivity for PCR in MB cases in other hosts, is usually given somewhere between 87% to 100% (Reibel, Cambau and Aubry, 2015; Fontes et al., 2018).

All ERS with clinical leprosy lesions described prior to this study have had MB LL or BL (Meredith *et al.*, 2014; Simpson *et al.*, 2015; Avanzi et al., 2016) and have tested positive for leprosy bacilli DNA. One would expect these cases to be easily diagnosed via PCR since the tissue sample was taken from the lesion itself. However, in this study samples were not taken from leprosy lesions in order to monitor the natural progression of the lesions. It was furthermore assumed that due to the previously described bilateral nature of leprosy lesions in ERS bacteria would still be present in both ears even if only one ear showed signs of a clinical lesion. As a result, samples were taken from the unaffected ear in ERS presenting with unilateral lesions. Of the six clinical cases of leprosy included in this part of the study, only two were positive for *M. leprae* DNA by PCR. *M. leprae* DNA was not detected in the remaining cases suggesting that the assumption based on bilateral observations may not be justified or that the bacterial load was too low to detect.

One could argue that the four ERS from which no *M. leprae* DNA was isolated may not be true leprosy cases but have visually similar lesions of a different origin. However, in three of the four ERS, leprosy specific αPGL-I ratios above the cut off for positivity were detected. All these ERS came from BI. It is thus likely that they are indeed true leprosy cases and that the positioning of the punch sampling site was responsible for the absence of *M. leprae* DNA.

The fourth ERS did not have any detectable αPGL-I. This ERS did not present with any lesions on the ears, only a single lesion on the right hock, resulting in a full body score of 4 (severity category 1). As αPGL-I levels are correlated with disease severity, they may be below the cut-off for positivity in such a mild case, and thus not allow for the same reassurance that is available for the other ERS. Whether this ERS was misdiagnosed clinically or the PCR is false negative cannot currently be determined. Resampling of the animal at a later point in time could provide clarity.

The two ERS with clinical lesions that had a positive PCR result both had severity category 4 lesions, one with a full body score of 27, the other of 30. In these ERS lesions were present on both ears and in one on both hocks, in the other on one hock and on the scrotum. It is thus easily conceived that these two ERS may have had higher bacterial loads, i.e. a small

tissue sample was more likely to contain bacteria than in the others. The PCR method used in this study is purely qualitative, not quantitative. It does thus not allow any judgement on the limit of detection for *M. leprae* DNA in ERS. In humans real-time quantitative PCR techniques are thought to offer increased detection rates, sensitivity and specificity in leprosy diagnostics compared to conventional PCR methods (Martinez *et al.*, 2014). Such methods could be explored for ERS as well to determine a minimum level of detection specific to this species. However, if bacilli are not evenly distributed in all tissues or within a tissue, false negative results are still possible even with the most sensitive diagnostic method. This subject is addressed in chapter 4 (p. 109).

It is likely that sampling the skin lesion itself would improve the level of detection. However, with a punch biopsy of the type used in this study this is not readily possible. Future studies could explore if sufficient material for molecular diagnostics could be collected from a fine needle aspirate from the lesion. It has been described in humans that fine needle aspirates are suitable for histological confirmation of the presence of AFB (Baddam *et al.*, 2018). It appears therefore realistic, that samples collected with this method would contain enough bacteria to allow a detection of their DNA via PCR. A fine needle aspirate would cause less trauma to the sensitive skin over the lesion, and the needle could be inserted from the relatively healthy skin at the outer border of the lesion and pointed towards the lesion centre.

Similar insecurities remain in other hosts where molecular methods may return false negative results based on sampling location, and false negative results can never be excluded with absolute certainty (Martinez *et al.*, 2014). It is has therefore been recommended that molecular methods in humans should always be combined with clinical assessment and serological tools to assess whether a true infection is present (Sousa Lima *et al.*, 2019) and in NBA serological methods are thought to be the most sensitive tool to detect leprosy cases, before both clinical and molecular methods (Truman, 2005; Sharma *et al.*, 2015).

Despite its shortcomings, PCR from ear punch tissue samples allowed the identification of ERS in which leprosy bacilli are present before they show signs of clinical disease in this study. It is thus a valuable tool in ERS leprosy diagnostics. It is not currently known whether non-clinical ERS in which *M. leprae* DNA can be detected play a role in sustaining leprosy within the squirrel population, and it is unfortunately beyond the scope of this study to address this point. Another important aspect is, whether all ERS in which *M. leprae* DNA is present will develop clinical leprosy later in life. This is explored in chapter 5 (p. 141).

3.4.3. Serological testing of ERS

αPGL-I

Serum samples

ERS do mount a humoral immune reaction to an infection with leprosy bacilli (Avanzi *et al.*, 2016). This study showed that the point of care αPGL-I UCP LFA is suited to measure this reaction and that the reaction is correlated to the presence of clinical signs of leprosy. Therefore, an ERS with both a positive αPGL-I results and clinical signs is very likely to be a leprosy case. αPGL-I is, however, not regularly elevated before the onset of clinical disease in this species, unlike in NBA, where serological methods are the most sensitive screening tool (Truman *et al.*, 1986; Truman, 2005; Balamayooran *et al.*, 2015).

αPGL-I can only offer information on the host humoral immune reaction to the presence of leprosy bacilli (van Hooij *et al.*, 2018). An earlier, cellular immune response to the presence of leprosy bacilli could be present, but would not be identified (Nath, Saini and Valluri, 2015; A. van Hooij *et al.*, 2016). In humans, monocyte produced IP-10 improves the ability to discern contacts from TT/BT cases (Geluk *et al.*, 2012), and acute phase protein, such as liver produced C-reactive protein (CRP), is elevated early in LL/BL (van Hooij *et al.*, 2018), thus allowing for an earlier detection of these cases. However, these markers did not correlate with the presence of leprosy bacilli DNA or clinical signs of leprosy in ERS, based on an unpublished trial carried out in collaboration with the leprosy serology experts in Leiden. Differences may exist in the cellular immune response of both species, or in the structure of the proteins involved, resulting in the test antibodies used on the human strips not binding to ERS proteins. Therefore, serological markers that could be used to identify leprosy infection before the onset of clinical symptoms in ERS would need to be investigated in a species-specific approach.

Being able to confirm a clinical leprosy diagnosis using the αPGL-I UCP LFA is still highly valuable on its own, particularly when considering that molecular methods were not reliable for confirming clinical disease under the conditions of this study. Being able to readily distinguish live leprous ERS from those with similar looking skin lesions caused by other conditions is highly relevant. It may result in further diagnostics identifying conditions that are actually treatable on a short timescale. It could also guide decisions and management of ERS that have been taken into human care, for example where they were found injured or trapped after lesions causing welfare concerns were observed.

ERS with clinical leprosy should not be released into the wild, when this would pose the risk of introducing leprosy bacilli into a population to which the disease or the particular strain of the bacteria is not endemic. They need to be excluded from translocation projects (Woodford, 2000). Release of animals affected by a chronic disease is always controversial, and often discouraged, especially when further public health implications exist. Rehabilitation

and release efforts in badgers are an example (Mullineaux, 2018). Having a point of care tool like the αPGL-I to confirm an infection is thus very important.

The correlation of clinical lesion severity and αPGL-I titre observed in ERS is a phenomenon also described in other hosts. The αPGL-I assay alone is much less sensitive in PB human cases (30-60% αPGL-I positive) than in MB cases (80-100% αPGL-I positive) (Lastória and de Abreu, 2014b). Thus, the αPGL-I UCP LFA has an increased potential for false negative results in mild cases that users need to be aware of. The current dataset does not allow to determine the exact time point from the onset of clinical disease at which αPGL-I levels can be expected to be above the threshold for positivity. While re-tests at a later point in time may provide clarity, they will not always be possible. If the clinical signs are very typical for early leprosy, it is better to be cautious and still treat such ERS as potential leprosy cases.

Unfortunately, no ERS infected with *M. lepromatosis* were available for inclusion in this study, and it was not possible to assess the ability of the αPGL-I UCP LFA to confirm an infection with this pathogen in ERS.

Blood drop and prick trial

The strong correlation of αPGL-I UCP LFA results from blood drop and serum imply that the two sample types can be used as equals in ERS.

This is a significant advantage for animal welfare, as it is not necessary to collect a whole blood sample, something that usually requires general anaesthesia in wild ERS. The small sample size is also of advantage where ERS that have suffered previous blood loss are concerned, as the blood volume needed is much smaller.

However, collecting a blood drop sample by pricking an ERS with a small needle, to reduce invasiveness and allow sampling of a conscious animal in a handling cone, proved difficult. Prick needles developed for use on human fingers were too small in diameter and were difficult to handle in ERS. The needle easily pierced right through a squirrel ear and correct positioning on the tail was not straightforward. It was found that in an anaesthetised squirrel a regular injection needle was safer to use as the handler could be permanently aware of where the needle tip was and guide the depth of the prick. Sufficient immobility to use such a needle would be difficult to guarantee in a conscious ERS.

The front footpad did not bleed at all following pricking and would put the handler at risk of being bitten by a conscious animal. On the ear hair residuals interfered with blood drop formation and due to the thinness of the ear there was a high risk of pricking through the ear and into the handler's hand. The hind footpad again never produced a large enough blood drop for sampling. The tail needed to be prepared extensively to be able to collect a blood drop here in at least a few ERS. The steps necessary are unlikely to be practical in a conscious ERS where sudden, uncontrolled movement of the tail could occur and the risk of degloving injuries exists when fixation of the tail is attempted.

Different ideas should be explored for the collection of usable blood drops in ERS. For example, Nobuto blood filter strips could be trialled, as these are successfully used in NBA (Loughry *et al.*, 2009). However, it would be necessary to assess if blood collected in this manner could be used for the αPGL-I UCP LFA.

Body cavity fluid

If the UCP-LFA had performed equally well in body cavity fluid as in live squirrel samples, it could have been used in passive surveillance efforts relying on opportunistic carcass samples. The latter is often the case where wildlife species are concerned. The use of body cavity fluids for leprosy diagnostics is not reported in the other host species.

Unfortunately, test sensitivity was reduced in this sample type and detected αPGL-I ratios lower in body cavity fluid compared to the other two sample types. Either only a small fraction of the αPGL-I diffuses out of the blood vessels post mortem and can be detected in body cavity fluid or αPGL-I breaks down quickly after the death of the animal, a process that cannot fully be halted by freezing the carcass. As animal welfare is no longer a concern post mortem and tissue samples can readily be collected at this point, molecular methods appear more reliable for diagnosing leprosy in ERS post mortem than measuring αPGL-I in body cavity fluids.

3.4.4. Diagnosing leprosy in live ERS

All methods explored here are highly valuable for ERS leprosy diagnostics. However, no single diagnostic method can identify all live ERS in which leprosy bacilli are present. The same is true for the other host species. In humans, the diagnosis is still primarily clinical, backed up by laboratory tests (Lastória and de Abreu, 2014a), while in NBA, where clinical signs of disease in wild specimen are rare, serological methods are preferred, often combined with molecular methods (Loughry *et al.*, 2009; Sharma *et al.*, 2015). In ERS it may make sense to use all three methods in some animals, but in others using two out of the three may be sufficient. This needs to be considered, particularly in the context of conservation efforts where funding is usually limited. The order in which tests should be run or whether they can be omitted may vary depending on why the leprosy status of a squirrel is being assessed. The main reasons for using leprosy diagnostics in an ERS are a) the observation of suspicious clinical lesions and b) surveillance or pre-translocation screening efforts. A thorough clinical assessment should form the basis of any leprosy assessment. Below effective test combinations are proposed in a diagnostic decision tree (Figure 37).

Aside from choosing the best test combination, defining a terminology that is logical and thus accessible to all those who would want to diagnose leprosy in ERS needs to be established to allow for consistent result reporting.

For the purpose of this thesis, the following definitions and diagnostic decision tree were proposed. They should also be valid to apply beyond this piece of work until further diagnostic methods and more information becomes available:

- **Leprosy case**: An ERS with clinical signs of leprosy as defined in 3.4.1. (p. 89) or lesions that match the lesions descriptions provided by other authors (Simpson *et al.*, 2015) with a leprosy specific αPGL-I titre above the cut-off for positivity, and/or where *M. leprae* or *M. lepromatosis* DNA is isolated. For future use, this isolation should be attempted from a sample of the lesion itself. Where this is not possible, false negative PCR results can occur.
- **Subclinical leprosy case**: An ERS without clinical signs of leprosy, in which *M. leprae* or *M. lepromatosis* DNA is isolated from a tissue sample, and an immune response to the pathogen can be detected. While they may exist, current methods are unlikely to be able to identify them.
- **Colonised squirrel**: An ERS without clinical signs of leprosy, in which *M. leprae* or *M. lepromatosis* DNA is isolated from a tissue sample, but no immune reaction of the host to the pathogen can be detected. Once appropriate markers to detect a specific cellular response to leprosy bacilli in ERS are identified, some animals that are currently placed here may be reclassified as subclinical cases.
- **Leprosy contact**: Any ERS from a population in which leprosy is endemic, even when no clinical sign of disease or bacterial DNA can be detected. If a αPGL-I titre above the cut-off for positivity occurs in such an animal, it is still classified as contact.
- **Leprosy unlikely, further tests necessary**: ERS with clinical signs similar to leprosy lesions, but a αPGL-I titre below the cut-off for positivity or serology is unavailable and in which no leprosy bacilli DNA is detected or PCR is not attempted. Further differential tests be performed or leprosy specific diagnostic tests repeated at a later point in time. Where the suspicion that this is a leprosy case based on the clinical assessment is very strong, these animals can also be called leprosy suspicious, further tests necessary.
- **Leprosy unlikely**: ERS without clinical signs of disease in which no leprosy bacilli DNA is detected and that originate from a population to which leprosy is not endemic.

The combination of diagnostic tests that should be used to reach each diagnosis are depicted in grey in the decision tree in Figure 37. The box of the αPGL-I serology when no skin changes are seen in the clinical assessment is white, as in this case the test is unlikely to offer reliable additional information.

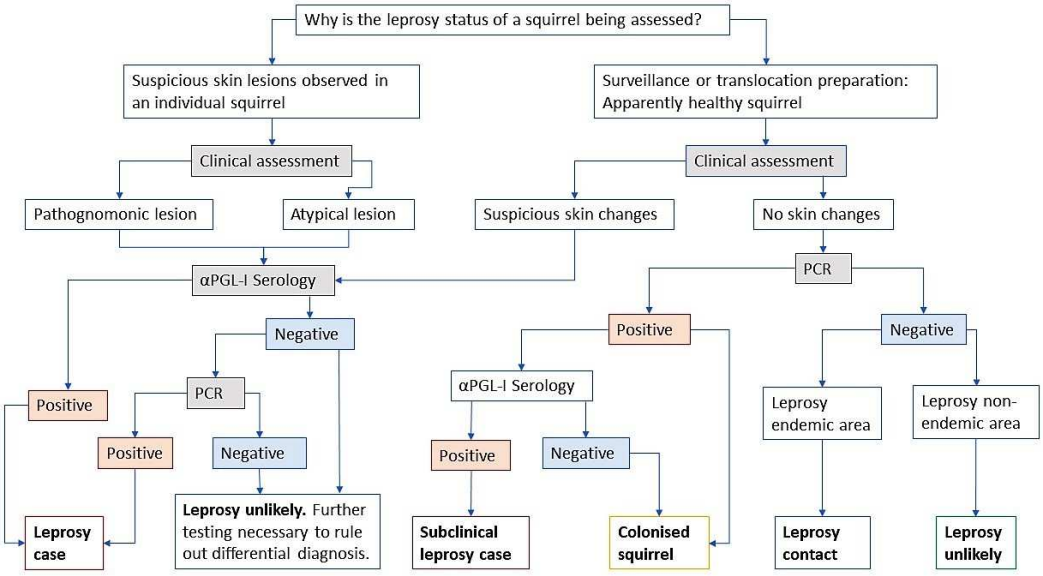


FIGURE 37: DIAGNOSTIC DECISION TREE TO ASSESS THE LEPROSY STATUS OF ERS

A focus of future research should be to develop diagnostic methods able to differentiate subclinical leprosy cases and colonised squirrels. Ideally, in developing diagnostic methods further they should be less invasive and adaptable for conscious ERS in a handling cone. This could allow for wider active surveillance, as sampling could then be more readily integrated in ongoing conservation projects.

Additional diagnostic options - future research

Fine needle aspirates (Baddam *et al.*, 2018) were already discussed on page 92 et seq.. They could be an option to collect material directly from a leprosy lesion without risking a large, potentially poorly healing injury that could result from a biopsy in the lesioned tissue. However, they provide a very small sample which may not always contain enough bacteria for PCR detection. Acid-fast staining of air-dried fine needle aspirates could also be attempted. Another way to add histological methods to live ERS diagnostics would be slit skin smears from lesions, where the potential problems of the slit injury can be successfully mitigated. However, they are only reliable when performed and interpreted by experienced personnel (Scollard *et al.*, 2006). Still they could have a role where clinicians do not have access to serological or molecular diagnostics, but want to know quickly whether a suspicious skin lesion in an ERS contains AFB. This could guide whether treatment of an ERS presenting with lesions should be attempted.

Additionally, it could be assessed whether the blood volumes collectable from ERS are suitable for molecular diagnostics. In humans, detection of *M. leprae* DNA is possible from 3 to 5ml blood samples in 70% of PB cases, 95.25% of MB patients, and in some (6.25%) household contacts. If smaller volumes were sufficient for a detection, it should be compared how test sensitivity in this sample type compares to the detection in tissue punch samples. If

sensitivity is similar or even improved, a single sample type could be used for PCR (cellular portion) and serological (serum) diagnostics, thus reducing the impact of sampling on the ERS.

It has also been shown that an infection with leprosy leads to differential expression of miRNAs in the lesion tissue and in blood samples of human patients (Singh, Singh and Chauhan, 2013; Salgado, Pinto, *et al.*, 2018). miRNAs are small (18-22 nucleotides) non-coding RNA molecules with the capacity of translational downregulation of messenger RNAs. Thus they have a role in regulating cell and tissue homeostasis and in the molecular pathogenesis of disease, probably by regulating the host immune response (Soares *et al.*, 2017). The expression of a large number of miRNAs has been shown in skin sections when household contacts were compared to leprosy patients, and within the leprosy spectrum and the reactional stages (Singh, Singh and Chauhan, 2013). In blood, differential expression of miRNAs has also been shown, but varied from the patterns observed in tissue sections of lesioned skin (Salgado, Pinto, *et al.*, 2018). miRNAs could serve as biomarkers of infection, to aid understanding of leprosy pathogenesis and as therapeutic targets once their role is fully understood. Consistent patterns of expression in leprous ERS would need to be identified and individual miRNAs validated for this purpose (Soares *et al.*, 2017). No research has been done in ERS in this area, but may be interesting in the search for additional diagnostic markers with the potential to identify early colonisation/infection, assuming that pathogen invasion alters host-cell homeostasis. Collecting samples with intact miRNAs will require sampling of live squirrels, but acquiring a sufficiently large tissue section from leprosy lesions may cause animal welfare concerns. Furthermore, the sample sizes would necessarily be low due to the mainly sporadic occurrence of clinical cases, thus the statistical power to identify significant differences in miRNA expression in this species may be limited. Samples from opportunistically collected carcasses may be of limited use for miRNA assessment. Individual miRNAs have variable degradation times which may lie anywhere between four hours and five days, sometimes longer (Gantier *et al.*, 2011; Marzi *et al.*, 2016). Therefore, while relatively fresh carcasses with a known time of death that could be factored into the analysis could be useful. Carcasses found in the wild where the time of death is unknown may not present with the same miRNA composition they had while the animal was alive. Collecting blood samples from ERS may be a more viable strategy, however, here the low volume (only up to 1ml at any one time) of the samples may again limit the amount of analysis that can be done. Waiting until miRNA biomarkers for leprosy are firmly established in humans and then using a targeted approach to assess the presence of similar expression patterns in ERS may be a more viable strategy than attempting to establish miRNA expression pattern in leprous ERS from scratch.

Conclusion

The clinical diagnosis of leprosy was successfully adapted to the presentations seen in live ERS, and a scoring and categorisation system for lesions proposed that allows disease progression in ERS to be followed over time and to infer how lesions influence ERS welfare. Molecular and serological tests were successfully adapted and validated for use in live ERS. It needs to be kept in mind that no true gold standard for the diagnosis of leprosy exists today across the host spectrum. Combining the diagnostic methods introduced in this chapter does allow the identification of cases with an improved level of confidence. However, the number of colonised ERS in a population may be underestimated and subclinically infected ERS may not be identified as such. The methods introduced here can be used in research, clinical and pre-relocation screenings alike, but are currently not commercially available. As refined and improved methods are developed in the future, they can be integrated into the diagnostic decision tree in accordance with their individual properties and assist in refining the terminology around ERS leprosy.

Chapter 4: Clinical and histopathological characterisation of leprosy in ERS

4.1. Introduction

Following the definition of leprosy lesions in ERS for diagnostic and classification purposes in chapter 3 (p. 89) the clinical presentation of leprosy in ERS can now be explored further.

Data presented in chapter 3 showed that the clinical presentation of leprosy can be more varied than the previously published literature had suggested (p. 70). Nevertheless, it was possible to propose a clear and simple definition (p. 89). This raised the question whether leprosy lesions in ERS are pathognomonic, i.e. characteristic enough so that the diagnosis can be made immediately where they are observed. While it is difficult to test this first hypothesis directly, given the fact that the full spectrum of leprosy lesions in ERS may not yet be known, it can be challenged indirectly, for example by assessing how often a clinical diagnosis made can be backed up with laboratory results and by comparing clinical signs observed in different leprous ERS.

A disease is not always just characterised by the specific clinical signs it induces in the host, but sometimes also by less specific, secondary changes, such as loss of body condition and weight or general ill health. In some chronic diseases such unspecific changes may precede typical clinical signs, one example being diabetes (Baumert *et al.*, 2014). In humans, such secondary changes do usually occur only after the onset of leprosy specific clinical signs, if at all (Lane *et al.*, 2006). It is not known whether leprosy in ERS is linked to any unspecific changes or if such could even be indicators of an early leprosy infection. Health parameters assessed in this study, such as BCS, weight or GHS can be used to explore this topic and to test the second hypothesis of this chapter, that leprosy has an impact on health indicators in individual ERS.

In humans and NBA's both sexes appear to be similarly at risk of an infection with leprosy bacilli and of developing leprosy, but some studies have found a higher proportion of males (human) or females (NBA) being infected (Morgan and Loughry, 2009; Gaschignard *et al.*, 2016). The dataset collected in this study offers the opportunity to test a third hypothesis: Leprosy is as frequently observed in male as in female ERS.

While unsuitable as a routine diagnostic tool for live ERS, histopathology has been pivotal in the initial identification of leprosy in ERS (Meredith *et al.*, 2014), and can provide some information not obtainable by other means. Most importantly this includes information on the inflammatory reaction the host shows in response to leprosy bacilli, allowing characterisation of leprosy lesions within the histological Ridley-Jopling spectrum (Ridley and Jopling, 1966). Currently, the spectrum of inflammatory reactions to leprosy infection in ERS is narrower than in other host species (see p. 10 and p. 17) and limited to BL and LL leprosy. In a

recently described condition this may not be a matter of true absence of other variations, but such variations may simply not have been observed yet. Therefore, histological assessment of lesions in ERS leprosy case carcasses was completed to assess whether new presentations could be observed. Thus, the fourth aim of this chapter is to assess whether the full spectrum of leprosy lesions described in other hosts is present in ERS as well.

Having several leprous ERS carcasses available does also allow to assess whether it is acceptable to rely on molecular over histological methods in live ERS, by comparing the presence of AFB and *M. leprae* DNA in different tissues. By addressing the fifth hypothesis of this chapter (molecular and histological methods are both suited to identify leprosy in ERS) it can be determined whether relying on molecular over histological methods in ERS diagnostics is sensible, or might result in cases being overlooked.

Leprosy can cause localised or systemic disease in humans and armadillos. For ERS it has been shown previously that leprosy bacilli DNA can occasionally be isolated from different skin areas, spleen, lung and liver tissue using molecular and histological methods (Avanzi *et al.*, 2016). In chapter 3 the ear was empirically chosen as tissue sampling site for molecular leprosy diagnostics (p. 72). While some colonised ERS were identified, it is impossible to say if any, or how many, were missed, or if another tissue would have offered different results or identified even more colonised ERS. By assessing the presence of AFB across a range of ERS carcass tissues and isolating *M. leprae* DNA from the same tissues the sixth hypothesis of this chapter that leprosy bacilli are present in a range of tissues in ERS is addressed. High bacterial loads are likely to make it easier to identify an infection with leprosy bacilli than low bacterial loads, a point proven by the continued difficulties in human medicine to identify PB leprosy cases before severe clinical signs are present (Lane *et al.*, 2006). The bacterial index, which can be determined in acid-fast stained tissue sections, was therefore used as additional parameter to determine the tissue best suited for leprosy diagnostics in ERS.

Addressing these six aims/hypotheses of this chapter will broaden the information base available on clinical and histological characteristics of ERS leprosy, identify if there are additional parameters that could aid leprosy diagnostics or allow to predict which ERS are more likely to be infected, and allow to further refine sampling for leprosy diagnostics in carcasses and live ERS.

4.2. Methods

4.2.1. Clinical presentation of leprosy in ERS

Information included in this chapter covers data from live ERS assessments and full post mortem examinations. For ERS seen live, a general health assessment was completed for each animal every time it was seen, as described in chapter 2 (p.43), and the presence/absence of leprosy lesions and their severity were noted in accordance with the scoring and categorisation system described in chapter 3 (p. 82). Blood samples were

collected from the femoral vein during each assessment and the αPGL-I UCP LFA performed to assess the presence of anti-leprosy bacilli antibodies described in chapter 3 (p. 75). Where ear tissue punches were collected, they were screened for *M. leprae* and *M. lepromatosis* DNA as described in chapter 3 (p. 72).

Opportunistically collected carcasses from BI and AR and additional carcasses that had been submitted to the UoE Red Squirrel Surveillance scheme were assessed and samples collected following the protocol introduced in chapter 2 (p. 46). Disposable scalpels and tweezers were used for all sampling and changed after every organ. Individual clean single use plastic petri dishes were used to separate organs and cut sections as necessary.

Where ante- and post-mortem information was available for an animal, the data was collated to provide the most comprehensive information (usually isolation of *M. leprae* DNA from tissue samples collected post mortem) and the animal was sorted into the session in which it was seen alive. Session numbers were kept in line with the other chapters of this thesis (1= autumn 2016 to 5 = autumn 2018) with an added session to describe animals seen only post mortem (6 = carcasses collected at different points in time). ERS with clinical lesions or a positive PCR for leprosy bacilli DNA were included, aiming to cover all ERS affected by leprosy, clinical leprosy cases as well as colonised squirrels (For definitions see chapter 3, p. 97 and Figure 37). Contact ERS without clinical signs of leprosy and from which no leprosy bacilli DNA had been isolated were used as “healthy” controls for the purpose of comparisons. The proportion of “healthy” ERS from the two populations included in comparisons was kept similar to the proportion of affected ERS from both populations. ERS of unknown PCR status without clinical signs of disease were excluded in this chapter.

As clinical disease and general state of the live animals changed over time, returning animals were included several times. Therefore, not all data in this set is independent. The purpose of this research effort was to describe the spectrum of leprosy lesions in ERS in as much detail as possible. Where the same individual ERS was seen several times, its condition and leprosy signs changed, and were thus relevant to be included again, artificially being treated as independent descriptions of leprosy lesions, to increase the available dataset. Chapter 5 will address these dependent samples as such.

Analysis

Data collected was summarised and analysed using R as specified under the individual subheadings below.

Leprosy lesions are pathognomonic. They are very similar between individuals.

Firstly, it was assessed in how many instances a clinical leprosy diagnosis could be backed up with a laboratory confirmation. Field and post mortem data were included. Secondly, samples from two carcasses (R3-17, R38-18) submitted to the UoE Red Squirrel Surveillance scheme under the suspicion that they might be infected with leprosy bacilli were

examined following the protocols of this study. Thirdly, the similarity of leprosy lesions seen in ERS throughout the study was assessed. To achieve this, the lesion scores and categories assigned to the different ERS were summarised, along with the location of the body in which lesions were observed. Images from the observed animals were grouped by category to illustrate the similarities and variations between lesions. Again, both live ERS and post mortem data of affected ERS were included.

Leprosy has an impact on ERS beyond the immediate clinical signs

The null hypothesis that BCS, weight and general health are similar in clinically diseased, colonised and contact ERS that are apparently unaffected by leprosy was tested using a Kruskal-Wallis test. Where significant differences between the three groups existed, an additional pairwise Wilcoxon rank sum test was run to determine between which groups the differences were statistically significant ($p< 0.05$). The data collected for comparison is ranked (BCS, GHS) or not normally distributed (weight). Only live assessed ERS were considered, as in ERS only assessed post mortem the presence of additional conditions impacting BCS, weight and GHS was highly likely.

Only ERS affected by leprosy (clinical case or colonised) were then considered to assess the effect of leprosy severity on BCS, weight and GHS. A Spearman's rank correlation was used to assess whether changes in BCS, weight or general health were correlated to the leprosy severity category. The different clinical categories of 1-4 and an additional category 0 for colonised ERS were used to represent severity.

Influence of ERS sex on proportion colonised by/infected with leprosy bacilli

The null hypotheses that the proportion of males and females colonised by leprosy bacilli or showing clinical signs of leprosy does not differ was tested using a chi-square test. Live and post mortem data was included in this analysis.

A Wilcoxon rank sum test was used to assess whether sex had an influence on disease severity represented by leprosy category (ranked data).

4.2.2. Histopathological and molecular assessment of leprosy affected ERS

Twelve carcasses were selected for detailed assessment following a pre-screening of ear tissue for *M. leprae/M. lepromatosis* DNA to identify animals in which leprosy bacilli were present. Ten animals with a positive PCR for *M. leprae* were selected and two negative controls. One of these subsequently was shown to carry *M. leprae* DNA, so that in total 11 PCR positive ERS, seven of which also showed clinical leprosy lesions, and one negative control were assessed in detail. No *M. lepromatosis* positive carcasses became available during the time of this study.

From each of the 12 carcasses the following tissues/organs were collected: ear, eye, nose, muzzle, mandibular lymph nodes, lung, front footpad, liver, spleen, intestines, kidney, hock

skin, hind footpad, mammary gland (females) and testicles (males). Details for the included ERS can be found in appendix III (p. 226). The ERS included in this part of the study were BIC001_16, BIC002_16, BIC003_16, BIC006_16, BIC007_16, BIC009_16, BIC010_16, BIC014_17, BIC016_17, BIC018_18, ARC016_18, and, as negative control, ARC017_18.

Sample collection started with skin samples, followed by the eyes and nose. Then the ventral neck skin was incised to collect the submandibular tissue, which included lymph nodes as well as saliva glands, connective tissue and fat. Only histologically confirmed lymph node tissue was later included in the analysis. The skin incision was then elongated along the ventral midline. In males, testicles were collected while dissecting off the skin. After assessing subcutaneous fat cover and the integrity of the abdominal muscles, the abdominal cavity was opened, beginning the incision at the sternum to the pelvis and cutting along the caudal rim of the ribcage to expose all abdominal organs. The spleen was removed first, followed by the gastrointestinal tract, liver, kidneys and adrenal glands. The diaphragm was then cut on the ventral edge, and the rib cage opened on the left. The muscles of the neck and lower jaw were cut, so that tongue, trachea, lungs and heart could be removed and separated. Testicles and mammary tissue were only assessed histopathologically and not via PCR. The muzzle was originally collected in two parts ("muzzle" and "scent glands corner of the mouth"), but as results did not vary between the two segments they were combined for analytical purposes. Not all organs were still present in all squirrels, depending on the state of decay and whether the animals had been predated. For paired organs the left one was fixed in 10% formalin for a minimum of 48 hours before trimming and the right in 70% ethanol. For unpaired organs representative sections were collected and stored as before (Figure 38, p. 107).

Histopathology

For histopathological assessment the fixed tissue and organ sections were trimmed and submitted for blocking and staining to the histology laboratory at Easter Bush Pathology, The (Royal) Dick School of Veterinary Studies, UoE. Up to four organs per animal were grouped for one block/slide, always following the same pattern (Table 16).

TABLE 16: TISSUES BLOCKED PER SLIDE

Slide 1	Slide 2	Slide 3	Slide 4
Ear	Hock skin	Muzzle	Scent glands
Eye	Nose	Mandibular lymph node	Front footpad
Hind footpad	Lung	Intestines	Spleen
Liver	Mamma/testes	Kidney	Other leprosy lesion

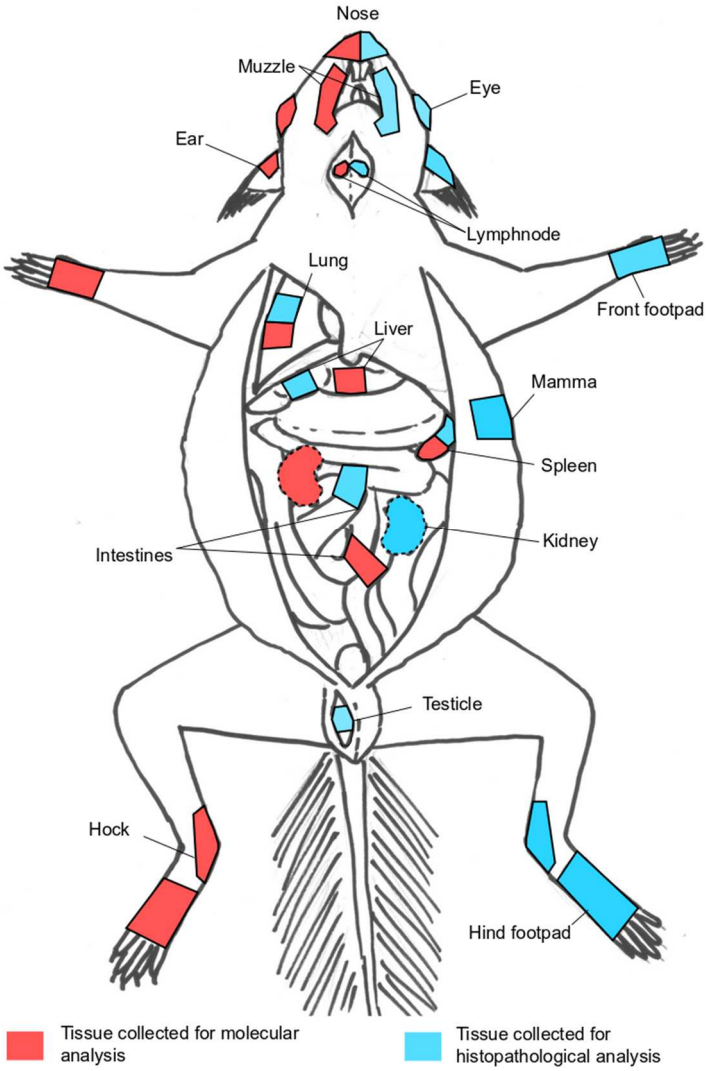


FIGURE 38: SQUIRREL SITUS, TISSUE/ORGAN SAMPLES COLLECTED FOR DETAILED MOLECULAR (RED, RIGHT BODY SIDE) AND HISTOPATHOLOGICAL (BLUE, LEFT BODY SIDE) ASSESSMENT ARE HIGHLIGHTED.

From each block two stained slides were requested, one stained in Ziehl-Neelsen (ZN) one in Haematoxylin & Eosin (HE). ZN staining has been used in all previous assessments for leprosy bacilli using ERS tissues (Meredith *et al.*, 2014; Simpson *et al.*, 2015; Avanzi *et al.*, 2016) with consistently good success. The laboratory used had previous positive experience in applying ZN staining to ERS tissues for leprosy diagnostics.

Slides were assessed using an Olympus BX41 light microscope at 20x and 40x magnification mounted with an Olympus DP72 camera, using Olympus cell imaging software for Life Science Microscopy. Each section was visually examined in full, moving through the section in tight zigzags. Presence/absence of AFB was documented, along with their number, given as bacterial index in accordance with the Ridley-Jopling classification (Ridley and Jopling, 1966, also see p. 10) and location in ZN stained sections. In H&E stained sections the presence/absence of an inflammatory reaction was noted. Where present, the

type of inflammation, and cell types involved were noted and, where appropriate, a category within the Ridley-Jopling classification (TT, BT, BB, BL, LL or InL) assigned. Observations made were discussed with an experienced veterinary pathologist prior to further analysis. For each section information on the presence/absence of macroscopic lesions was also available.

During the histological assessment of the ZN and H&E stained tissue sections notes were kept on location and state of bacteria, the tissue itself and on cell types forming the inflammatory lesions seen. These notes are summarised in appendix IV (p. 235).

Molecular diagnostics

From all available organs of the 12 focus carcasses a two to three mm cube of tissue (approx. 15mg) was cut in a sterile petri dish with a single use scalpel and the ethanol allowed to evaporate while the rest of the sample was returned into 70% ethanol for continued storage. The tissue cube was cut into 5-6 smaller pieces and DNA extraction carried out following the same protocol detailed in chapter 3 (p. 73 et seqq.). The only adjustment made was the use of 80µl AE buffer in the final elution step.

PCR amplification and amplicon visualisation were carried out following the protocols detailed in chapter 3 (p. 73 et seqq.). For the two animals from AR, and for one from BI two amplifications were run for each organ, using both LPM 244 and RLEP 7 and 8, to ensure that a co-infection with *M. lepromatosis* was not overlooked. As *M. lepromatosis* has never been isolated on BI and all results for the pilot animal were negative, the other samples originating from BI were only tested for the presence of *M. leprae* DNA.

Analysis

The whole histological spectrum of leprosy lesions is present in ERS

The Ridley-Jopling categories established by classifying the inflammatory reactions in leprosy lesions in H&E stained sections in conjunction with the BIn determined from the ZN stained section of the same lesion were used to assess whether the whole spectrum of leprosy lesions is present in ERS.

There is no difference in the ability to identify ERS leprosy cases using molecular and histopathological methods

To compare the performance of the histological and molecular methods, PCR results and detection of AFB were compared for tissues for which both were available.

A 2x3 contingency table was created to see how many tissues tested positive with one of the two or both tests. A Fisher's exact test was used to assess the null hypothesis that the probability of a positive result is the same regardless of the diagnostic method being applied.

There is no difference in the ability to identify ERS leprosy cases using a range of different tissues

It was summarised in how many of the eleven assessed ERS each organ was positive in the histological and molecular assessment, testis and mamma only included for histological assessment. Not all organs were available from all ERS. Thus, the proportion of each available organ positive for leprosy in molecular and histological diagnostics is presented as well. As “other leprosy lesions” were only present in three animals, and located in different areas of the body, they were not included in this analysis

To assess the difference in the ability to identify leprosy bacilli in the specific tissues using molecular and histopathological methods 10 tissues available from all ERS were used to create a 2x3 contingency table for each organ. Based on the positive results for both tests, the proportion of animals was calculated that would have been identified as a leprosy case had only one specific organ been used.

The BIn documented was used to determine which tissue/organ was most likely to harbour large numbers of bacteria, i.e. had a high BIn in most assessed carcasses. Additionally, it was assessed whether the distribution of leprosy bacilli through the ERS body was influenced by disease severity. To achieve this, the ERS were split into two groups, severely affected ERS (category 4; n= 6) and colonised or mildly affected ERS (category 0 and 1; n= 5). Due to the small sample size and variation within the sample, analysis of these results was purely descriptive.

4.3. Results

All ERS identified as affected by leprosy in this part of the study were affected by *M. leprae*.

4.3.1. Clinical characterisation of leprosy in ERS

A total of 55 assessments of ERS affected by leprosy were available. Twelve were only assessed post mortem (n_{BI}= 10, n_{AR}= 2). Only one carcass was a juvenile, all other assessments were made in adult ERS. Forty-three assessments were completed in live ERS (n_{BI}= 41, n_{AR}= 2), ear tissue punches for PCR were collected during 22 of them. For four ERS additional information was added following post mortem assessment (n_{BI}= 3, n_{AR}= 1).

Leprosy lesions are pathognomonic. They are very similar between individuals.

Clinical skin lesions meeting the definition for leprosy lesions (p. 89) were seen in 34 assessments (30 live, four carcasses). PCR results were available for 15 of these animals and in nine (four carcasses, five live) the presence of *M. leprae* DNA was confirmed, while no bacterial DNA could be isolated from four (all live). Three of these, however, had a positive αPGL-I serology result to confirm them as leprosy cases.

αPGL-I serology results were available for 30 (live) out of the 34 assessments with clinical lesions. Three of these were negative, two in animals with very small and early lesions in just

one body area, and one with very early lesions in four body areas, and thus a higher score and category. Seropositivity is linked to lesion intensity and a re-test at a later point in time may have a positive result. Unfortunately, a PCR result was only available for one of these three animals. It was negative as well, placing this animal as leprosy unlikely if the decision tree (p. 99) is strictly followed.

Still, currently out of 34 clinically suspected leprosy cases only three (8.8%) could not immediately be confirmed using molecular or serological methods, with no PCR information available for two of them. In 91.2% the clinical diagnosis was confirmed, even with incomplete PCR data.

An additional consistent observation made in post mortem examinations and in one animal that had to be euthanised due to severe welfare concerns in autumn 2017, was that the cut surface of a fresh leprosy lesion is usually slightly yellow, bulks outward over the cut surface, and retains the firm rubbery texture (Figure 39).

FIGURE 39: LEPROSY LESION ON SQUIRREL PINNA REMOVED IMMEDIATELY AFTER HUMANE EUTHANASIA. THE TEXTURE OF THE LESION IS FIRM-RUBBERY AND THE CUT SURFACE OF THE LESION IS BULGING AND SLIGHTLY YELLOW

M. leprae DNA was isolated from all 21 animals without clinical lesions included here (8 carcasses, 13 live). All of the 13 squirrels seen live from which serum was available had a negative αPGL-I result, and were thus classed as colonised. None of these animals showed any skin changes in the clinical examination.

In neither of the two ERS submitted to the UoE Red Squirrel Surveillance scheme (R3-17, R38-18) under the suspicion of being leprosy cases were leprosy bacilli DNA or AFB detected. R3-17 presented with multiple bulbous skin lesions predominantly around eyes and a diffuse swelling with some hair loss of the chin/throat area. Smaller lesions, again with incomplete hair loss were observed on ears and right forearm. The surface of the lesions

was dull, and the skin came off in some areas when touched (Figure 40). When cut, the lesions were not bulking over the cut surface and were shiny, wet but not runny, and off white to pale yellow (Figure 41). In the histopathological assessment, this squirrel was diagnosed as a case of atypical histiocytosis (Figure 42).

FIGURE 40: CLINICAL LESIONS SEEN IN R3-17. INCOMPLETE HAIR LOSS TO SWELLINGS ON EYELIDS, CAUDAL RIM OF THE EAR AND DIFFUSE SWELLING WITH THINNING OF THE COAT ON CHIN AND THROAT

FIGURE 41: CUT SURFACE OF THE CHIN LESION OF R3-17

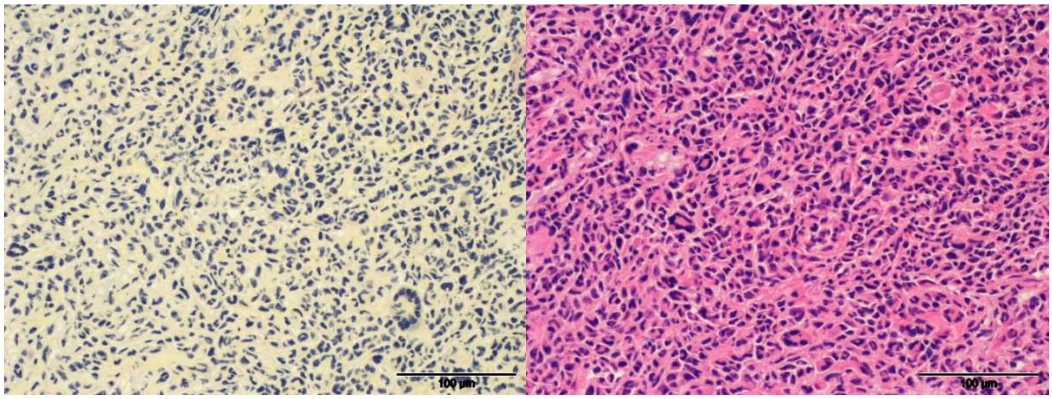


FIGURE 42: ZN (LEFT) AND HE (RIGHT) HISTOLOGICAL IMAGE OF THE CHIN LESION OF R3-17 SHOWING ABSENCE OF AFB AND PRESENCE OF ATYPICAL ROUND CELLS AND MULTINUCLEATED GIANT CELLS AS DESCRIBED FOR ATYPICAL HISTIOCYTOSIS (Smith *et al.*, 2017)

R38-18 had presented with an areas of hair loss and moderate skin thickening over the dorsal aspect of the nose and nostrils. No abnormal changes were observed on the skin of ears and hocks. No other lesions were seen on the animal, but it failed to thrive even under human care and was eventually euthanised. No obvious singular cause for its ill health could be identified (Figure 43). No leprosy bacilli DNA could be isolated from ear or nose lesion of this ERS. The animal was a very young adult male. As the size of the lesion was fairly large, this would be unexpected in a leprosy case of this age, where the disease is usually still non-clinical.

FIGURE 43: NOSE LESION OBSERVED IN R38_18 ANTE MORTEM.

To assess lesion similarity between leprosy cases the lesion scores assigned to all animals during clinical assessment (total n= 55) were used. ERS without lesions (n= 21) were assigned a score of 0. The minimum score where lesions were present was two, i.e. when a lesion is present it will at the very least be assigned one point for size and one for its character (p. 82 et seqq.). Scores for observed lesions (n= 34, all BI) were scattered between two and 51, with very few animals with lesions being assigned the exact same score. The most frequent score of 4 was assigned four times, a score of 2 was assigned thrice. Six score values were assigned twice and the remaining 15 values were only assigned to one animal each (Figure 44). This shows that clinical sign intensity and thus visual presentation is variable between individuals.

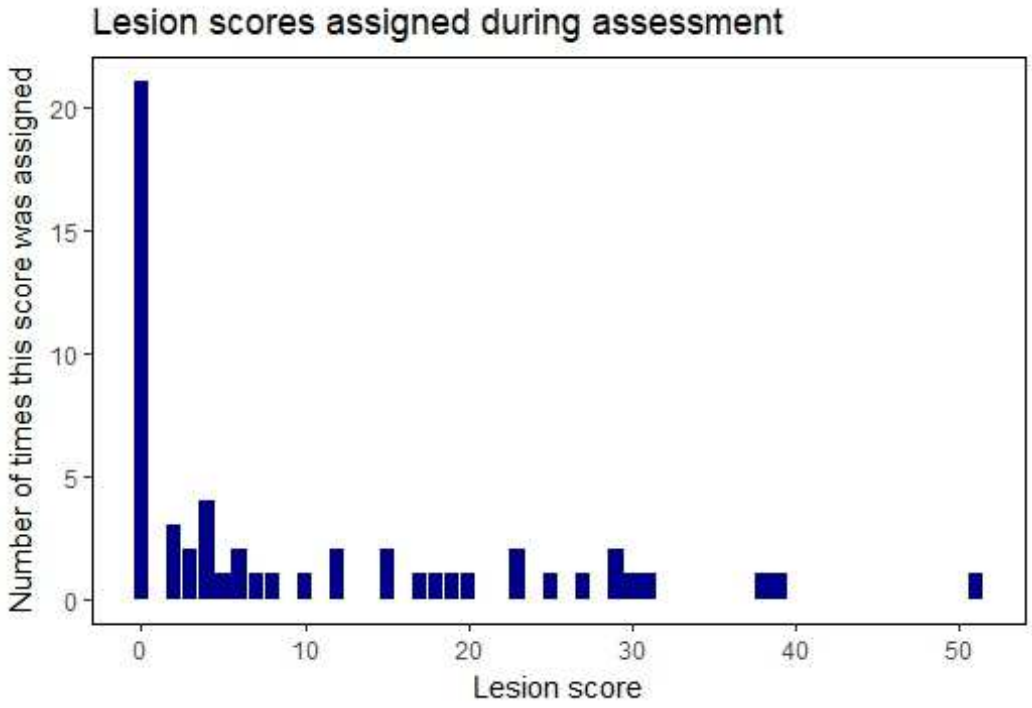


FIGURE 44: NUMBER OF TIMES (Y-AXIS) EACH NUMERICAL, ADDITIVE LESION SCORE (X-AXIS) WAS ASSIGNED TO AN ERS AT THE TIME OF ASSESSMENT. A SCORE OF 0 WAS ASSIGNED WHEN NO LESIONS WERE PRESENT. THE OTHER SCORES WERE CALCULATED AS DETAILED IN CHAPTER 3

Where clinical lesions were seen they were mainly classed as either mild (category 1, 32.4%) or severe (category 4, 47%). Mild to moderate (category 2, 14.7%) or moderate (category 3, 5.9%) lesions were observed much less frequently (Figure 45).

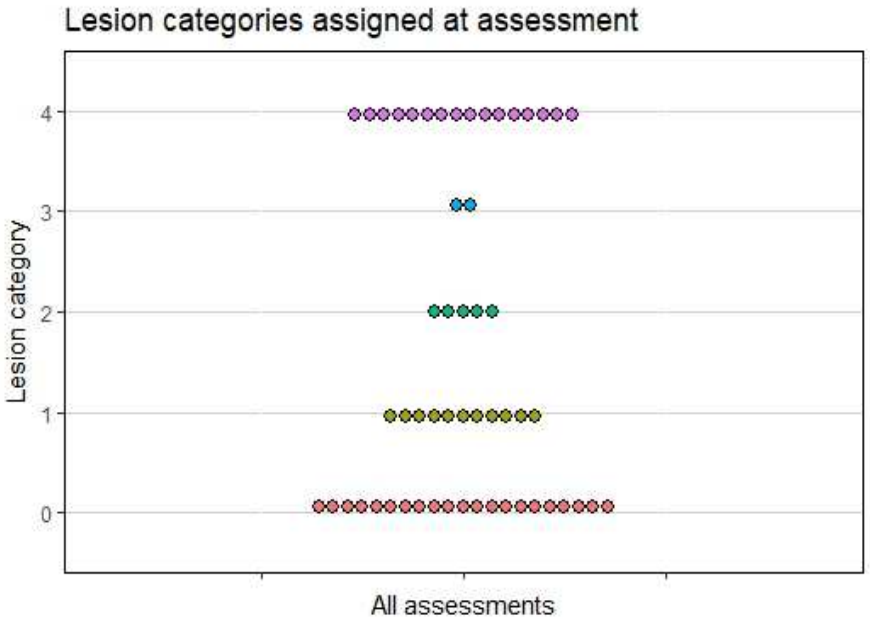


FIGURE 45: CATEGORIES ASSIGNED TO LEPROSY AFFECTED ERS. LESION CATEGORY 1= MILD, CATEGORY 2= MILD-MODERATE, 3= MODERATE, 4= SEVERE; CATEGORY 0 INCLUDES THE COLONISED ANIMALS WITHOUT CLINICAL SIGNS. EACH DOT REPRESENTS ONE ANIMAL

There was variability as to where lesions first appeared, the number of body areas affected, and whether lesions were bilateral, or unilateral, even within the same severity category. While fundamental characteristics like shiny appearance, hair loss and firm rubbery consistence were present in all cases, there were marked differences in the appearance of lesions seen in individual squirrels (Figure 46, Figure 47, Figure 48, and Figure 49).

FIGURE 46: EXAMPLES ILLUSTRATING THE DIVERSE FORMS MILD LESIONS (CHAPTER 3, P. 82 ET SEQQ.) CAN TAKE

FIGURE 47: EXAMPLES ILLUSTRATING THE DIVERSE FORMS MILD-MODERATE LESIONS (CHAPTER 3, P. 82 ET SEQQ.) CAN TAKE

FIGURE 48: EXAMPLE ILLUSTRATING A MODERATE LESION (CHAPTER 3, P. 82 ET SEQQ.)

FIGURE 49: EXAMPLES ILLUSTRATING THE DIVERSITY OF SEVERE LESIONS (CHAPTER 3, P. 82 ET SEQQ.)

Lesions were most often observed on the ears (94.1%). Only in two ERS with clinical leprosy lesions were no lesions noted on the ears. Eighteen ERS had lesions on both ears, eight just on the left and six just on the right ear. The hocks were the second most common location for leprosy lesions (22 out of 34 ERS, 64.7%), with 17 ERS having lesions on both hocks, four only on the left, and one only on the right. Both ERS that did not have lesions on the ears did have hock lesions. Less common locations for leprosy lesions were the nose and eyelid (two out of 34 ERS each, 5.9%). A swelling of a front footpad that was thought to be a leprosy lesion based on its texture was only seen in a single carcass. *M. leprae* DNA was detected in this footpad (BIC007_16, 2.9%; Figure 50). In three out of 17 male ERS with

leprosy lesions, scrotal lesions were observed (17.6%), making this the third most likely location for leprosy lesion in this sex.

FIGURE 50: SWELLING OF THE FRONT FOOTPAD IN BIC007_16

Leprosy has an impact on ERS beyond the immediate clinical signs

Forty-three assessments of ERS affected by leprosy, 30 clinically diseased ERS (all BI) and 13 colonised ERS (n_{BI}= 41, n_{AR}= 2), as well as, 44 assessments of live ERS unaffected by leprosy (n_{BI}= 42, n_{AR}= 2) were included to compare the effects of leprosy on the three chosen condition indicators. Only two ERS were selected for inclusion in the unaffected by leprosy group from AR, as squirrels in this population tend to have a higher weight than ERS on BI. Therefore, having a higher proportion of ERS from AR in the unaffected group than in the affected group could result in an apparent effect on the chosen parameters that are not truly linked to leprosy but to inherent traits of the population the ERS came from. Keeping the proportion of ERS from both populations similar for both groups should avoid this problem.

Most ERS were in normal (58.6%) or thin (40.2%) body condition. Only one ERS showing clinical signs of leprosy was emaciated (1.2%). Out of the thirty ERS clinically affected by leprosy 19 (63.3%) were in normal body condition, ten (33.3%) were thin, and one, as mentioned, emaciated. Nine (69.2%) of colonised ERS were in normal body condition, and four (30.8%) were thin. Twenty-three ERS unaffected by leprosy (52.3%) were in normal body condition and 47.7% (n= 21) were thin. It is possible to observe differences in body condition between contact, colonised and clinically diseased ERS as they are present in the sample set if the null hypothesis of no difference between the three groups were true (p= 0.4963, Figure 51).

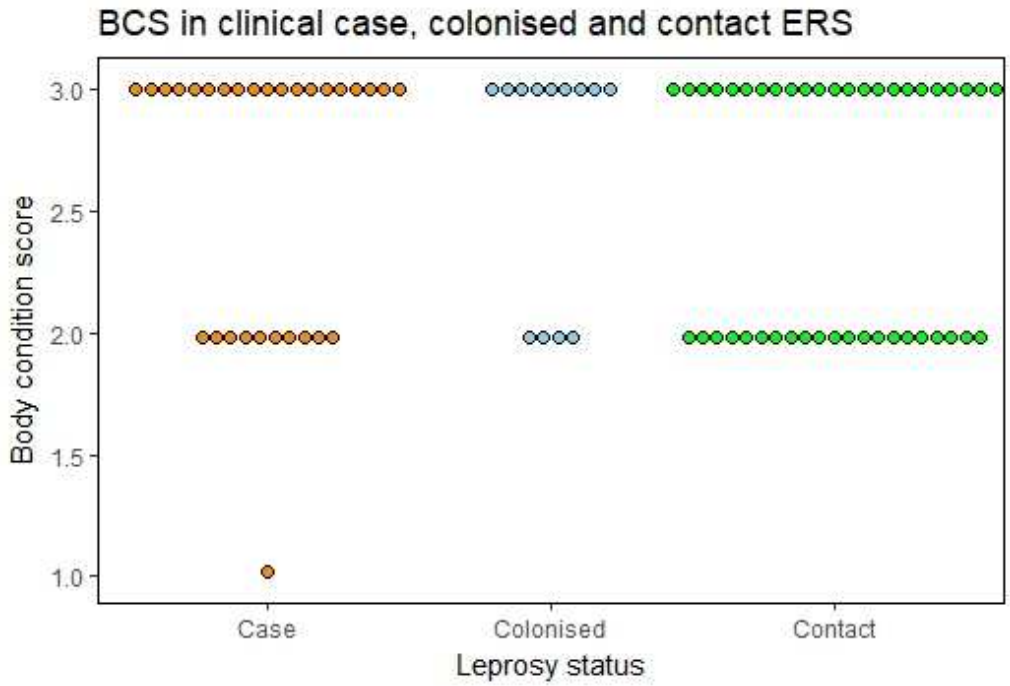


FIGURE 51: BCS OF ERS (CLINICAL CASES = ORANGE, COLONISED= BLUE, CONTACT= GREEN) AT THE TIME OF ASSESSMENT. BCS 1= EMACIATED, BCS 2= THIN, BCS 3= NORMAL.

ERS unaffected by leprosy had a mean body weight of 306.6g (SD= 19.0g, Min= 265g, Max= 370g). ERS affected by leprosy were on average heavier. Colonised ERS had a mean body weight of 319.2g (SD= 42.4g, Min= 270g, Max= 390g), and ERS with clinical leprosy lesions had a mean body weight of 327.2g (SD= 25.0g, Mon= 280g, max= 390g).

The differences in the average body weight between contact, colonised and clinically diseased ERS are unlikely to occur by chance alone. The average weight increases from contact over colonised to case ERS (Kruskal-Wallis test, p= 0.002887, Figure 52). While the difference observed between contact and colonised ERS (Wilcoxon rank sum test, p= 0.6124) and colonised and case ERS (p= 0.4793) were not statistically significant, the difference between contact and case ERS is statistically significant (p=0.0008).

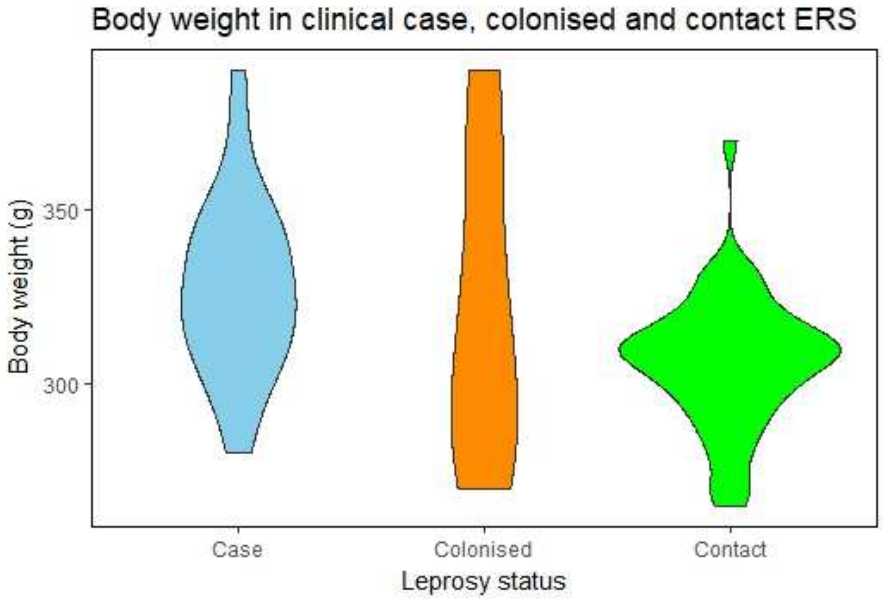


FIGURE 52: RANGE OF BODY WEIGHTS OBSERVED IN ERS AFFECTED BY LEPROSY (CASE= BLUE, COLONISED= ORANGE) AND UNAFFECTED BY LEPROSY (CONTACT= GREEN).

Most ERS were in good health (GHS category 1) at the time of assessment (n_{Total}= 64 (73.6%); $n_{contact}$= 35 (79.5%); $n_{colonised}$= 7 (53.8%); n_{case}= 22 (73.3%)). Some had minor or old injuries (GHS category 2) while still generally being in good health (n_{Total}= 18 (20.7%%); $n_{contact}$= 9 (20.5%%); $n_{colonised}$= 5 (38.5%); n_{case}= 4 (13.3%)).

One (7.7%) colonised ERS was chronically unwell but able to cope (GHS category 5). One (3.3%) ERS showing clinical signs of leprosy was sorted into GHS categories 3, 4, 5, and 6 each (3= acutely unwell, improvement likely, 4= acutely unwell, improvement unlikely, 6= chronically unwell, unable to cope).

Looking at the general health status of contact, colonised and clinically diseased animals, the null hypothesis that there is no difference between the groups cannot be rejected (Kruskal-Wallis test, p= 0.166; Figure 53).

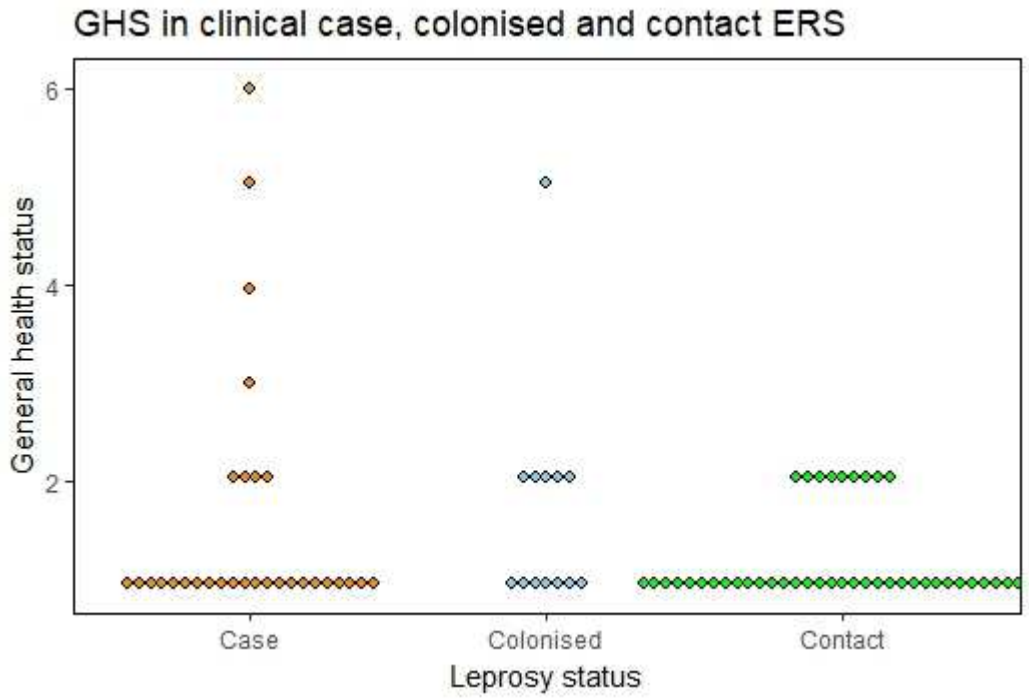


FIGURE 53: GHS OF CLINICAL CASE (ORANGE), COLONISED (BLUE) AND CONTACT (GREEN) ERS. GHS 1= IN GOOD HEALTH; GHS 2= IN GOOD HEALTH WITH MINOR OR OLD/HEALED INJURY; GHS 3= ACUTELY UNWELL WITH GOOD PROGNOSIS; GHS 4= ACUTELY UNWELL, UNLIKELY TO IMPROVE; GHS 5= CHRONICALLY UNWELL, COPING; GHS 6= CHRONICALLY UNWELL, NOT COPING

In the next analysis only ERS affected by leprosy (case and colonised ERS) were considered. When the observed body condition is split by severity category, four out of 13 colonised ERS (30.8%) were thin, and the remaining 69.2% in normal body condition. ERS with mild leprosy lesions (n= 10) were also thin (20%) or in normal body condition (80%). All ERS with mild to moderate leprosy lesions (n= 5) were in normal body condition. ERS with moderate leprosy lesions (n= 2) were in normal body condition (50%) or thin (50%). There appears to be a mild tendency of severely affected ERS (n= 13) to be in a lower body condition. One was emaciated (7.7%), seven thin (53.8%), and five in normal body condition (38.5%). However, the null hypothesis that there is no correlation between body condition and leprosy severity categories cannot be rejected (Spearman's rank correlation, p=0.091, rho= -0.26; Figure 54).

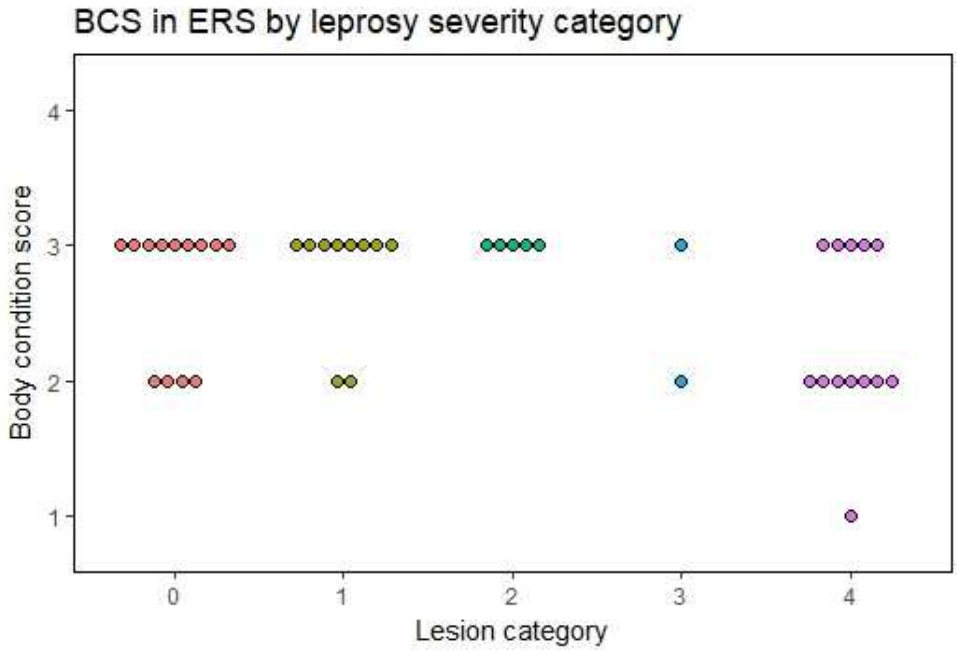


FIGURE 54: NUMBER OF ANIMALS WITH A CERTAIN BCS (Y-AXIS) IN EACH LEPROSY SEVERITY CATEGORY (X-AXIS: 0= COLONISED, NO CLINICAL LESION, 1= MILD, 2= MILD TO MODERATE, 3= MODERATE, 4= SEVERE)

Colonised ERS had an average weight of 319.2g (SD= 42.4g, Min= 270g, Max= 390g). ERS with mild leprosy lesions had an average weight of 313.9g (SD= 23.8g, Min= 280g, Max= 350g). ERS with mild-moderate lesions were on average slightly heavier with 327.0g (SD= 30.1g, Min= 300g, Max= 375g). Only two ERS were seen with moderate lesions. Both weighed 330g. ERS with severe lesion were again slightly heavier with 336.2g (SD= 23.6g, Min= 300g, 390g). Despite this tendency towards the higher weight in ERS more severely affected by leprosy, the differences are not statistically significantly correlated with the severity of observed leprosy lesions (Spearman's rank correlation, p=0.069, rho= 0.28) (Figure 55).

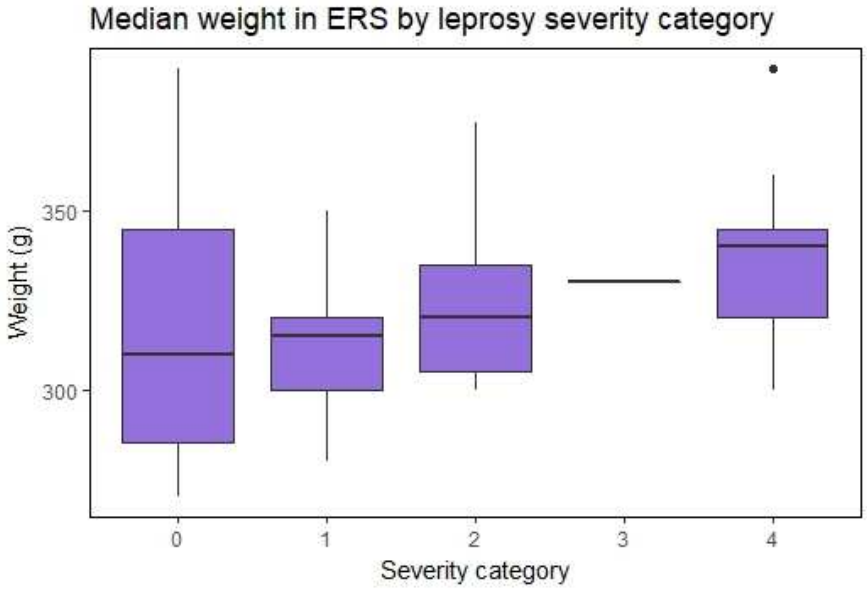


FIGURE 55: MEDIAN WEIGHT OF ERS ASSIGNED TO THE DIFFERENT SEVERITY CATEGORIES OF LEPROSY (0= COLONISED, 1=MILD LESION, 2= MILD-MODERATE LESION, 3= MODERATE LESION, 4= SEVERE LESION)

Colonised ERS (n= 13) were either in good health (53.8%), in good health with minor or old injuries (38.5%) or chronically unwell but able to cope (7.7%), as mentioned above. All ERS with mild, mild-moderate and moderate leprosy lesions were in good health or in good health with minor or old injuries (mild lesions: 80% GHS cat.1, 20% GHS cat. 2; mild-moderate lesions: 80% GHS cat. 1, 20% GHS cat. 2; moderate lesions: 50% GHS cat. 1, 50% GHS cat. 2). Nine (69.2%) ERS with severe leprosy lesions were still otherwise in good health. One (7.7%) ERS with severe leprosy lesions was classed in GHS categories 3, 4, 5, and 6 each.

The severity of leprosy lesions is still not statistically significantly correlated to the GHS category that the ERS was assigned to (Spearman's rank correlation, p= 0.8241, rho= -0.0349) (Figure 56).

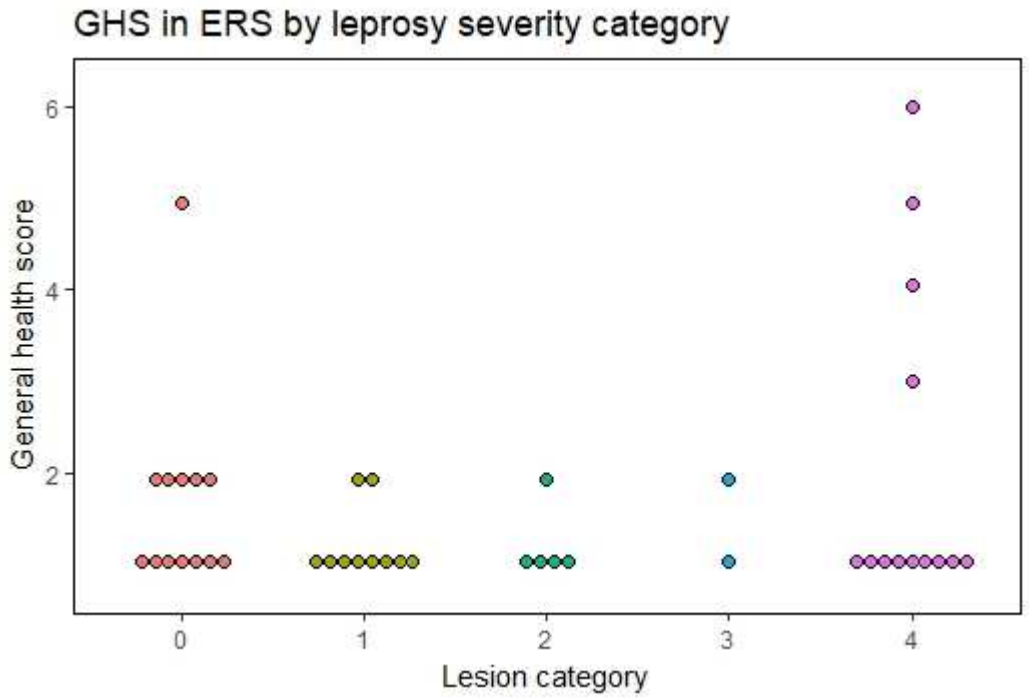


FIGURE 56: ERS ASSIGNED TO EACH GHS CATEGORY (Y-AXIS: GHS 1= IN GOOD HEALTH; GHS 2= IN GOOD HEALTH WITH MINOR OR OLD/HEALED INJURY; GHS 3= ACUTELY UNWELL WITH GOOD PROGNOSIS; GHS 4= ACUTELY UNWELL, UNLIKELY TO IMPROVE; GHS 5= CHRONICALLY UNWELL, COPING; GHS 6= CHRONICALLY UNWELL, NOT COPING) IN RELATION TO THE SEVERITY OF THEIR LEPROSY LESIONS (X-AXIS: 0= COLONISED, NO CLINICAL LESION, 1= MILD, 2= MILD TO MODERATE, 3= MODERATE, 4= SEVERE)

Influence of ERS sex on proportion colonised by/infected with leprosy bacilli

Out of the 55 ERS affected by leprosy assessed live or post mortem, 12 male ERS were colonised by leprosy bacilli and 17 were observed with leprosy lesions. Nine female ERS were colonised by leprosy bacilli and 17 clinically diseased (Figure 57).

This distribution is likely (chi-square test, p= 0.8122) when the null hypothesis of no difference in the occurrence of clinical lesions between the two sexes is true. When further dividing the dataset based on severity category in the two sexes the picture does not to change (Wilcoxon rank sum test, p= 0.71, Figure 58). Based on this dataset leprosy appears to affect both sexes evenly and the chances of an ERS being found in a certain severity category are not linked to sex.

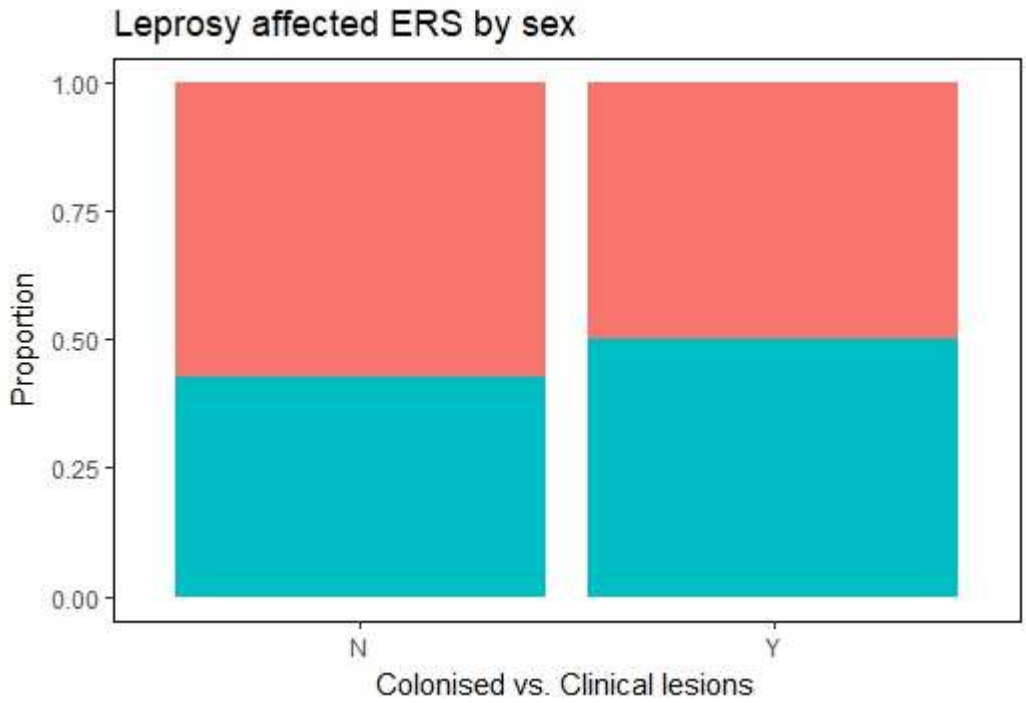


FIGURE 57: ERS COLONISED (N) BY M. LEPRAE OR SHOWING CLINICAL SIGNS OF LEPROSY (Y) BY SEX (BLUE= FEMALE, RED=MALE).

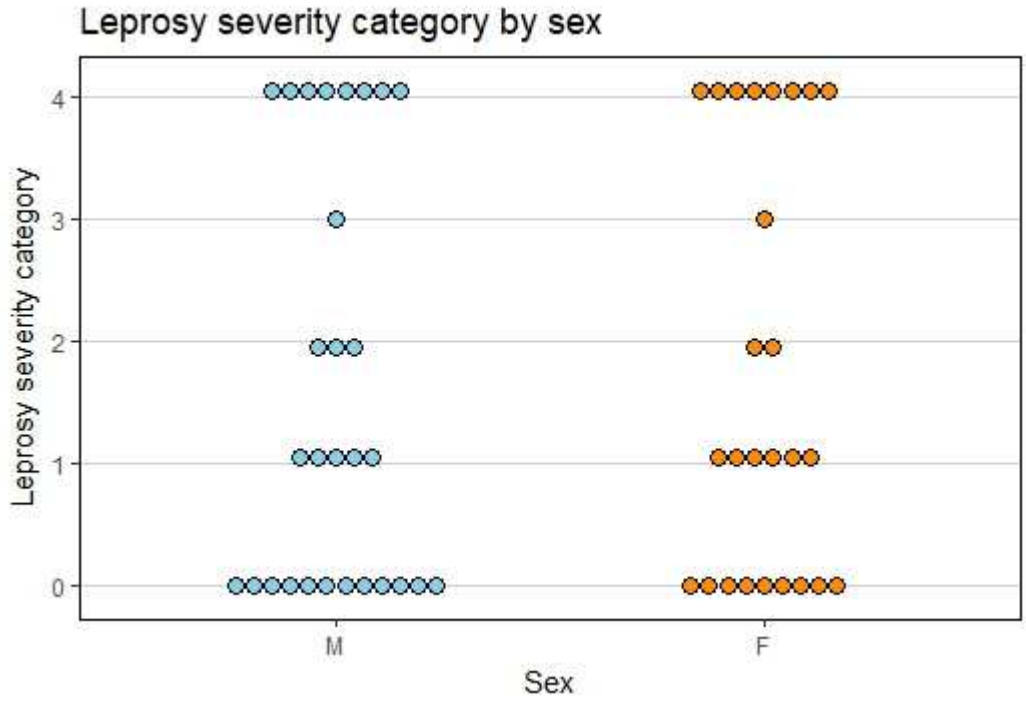


FIGURE 58: LEPROSY SEVERITY IN MALE (M) AND FEMALE (F) ERS

4.3.2. Histopathological and molecular assessment of leprosy affected ERS

Samples were available from 11 ERS carcasses affected by leprosy. All animals were adults, four were male (three with abdominal, one with scrotal testes) and seven female (three inactive, two in oestrus, one pregnant and one lactating). Seven showed clinical signs of leprosy (two male, five female). Most (n=6) had severe lesions, their additive point scores ranging from 15 to 51. In one ERS mild lesions with a point score of 4 were recorded. All these animals, as well as three animals colonised by *M. leprae* came from BI. Another ERS colonised by *M. leprae* came from the AR population. The number of each organ available for histological and molecular assessment along with the number of animals in which macroscopic lesions were seen in these particular organs is summarised in Table 17.

TABLE 17: NUMBER OF SPECIMEN AVAILABLE FOR EACH ORGAN TYPE FOR HISTOPATHOLOGICAL AND MOLECULAR ANALYSIS; NUMBER OF ERS IN WHICH THESE SPECIMEN SHOWED MACROSCOPIC LESIONS INDICATING LEPROSY

Sample type	No. available for histology	No. available for PCR	No. with macroscopic leprosy lesions
Ear	11	11	6
Eye	10	9	0
Nose	11	11	3
Muzzle	11	11	0
Mandibular lymph node	6	9	2 (enlarged)
Lung	10	10	0
Front footpad	11	11	0
Liver	11	11	0
Spleen	11	11	0
Intestines	11	11	0
Kidney	11	11	0
Hock skin	11	11	7
Hind footpad	11	11	0
Mammary gland	7	0	0
Testicle	4	0	0

Note that macroscopic lesions were only observed in a limited number of skin tissues and, in form of swelling, in the mandibular lymph nodes but not in internal organs. Other leprosy lesions (a small intranasal mass, a scrotal lesion and a lesion on the scalp) were available from three animals. Again, two of these were located in the skin. From all three *M. leprae* DNA was isolated. Only the scalp and scrotal lesions were assessed histologically as not enough of the intranasal mass was left. In the section of the scalp lesion assessed no AFB or signs of inflammation were present, while the scrotal lesion was classed as LL with a BIn of 5.

The whole histological spectrum of leprosy lesions is present in ERS

It was possible to classify lesions in four of the 15 tissues collected per ERS, namely ear, hock skin, nose, and one scrotal lesion, using the Ridley-Jopling system. Other tissues like

footpads or muzzle to which the system would in theory be applicable to did not show lesions.

An inflammatory reaction was observed in all 11 ear sections, nine hock sections, six nose sections and the scrotal lesion. One ear section could not be classified due to advanced autolysis. In one hock skin section the inflammation seen was classed as non-lepromatous and in another one no final decision could be made whether the inflammation seen was non-lepromatous or a very early stage of a lesion (Figure 59).

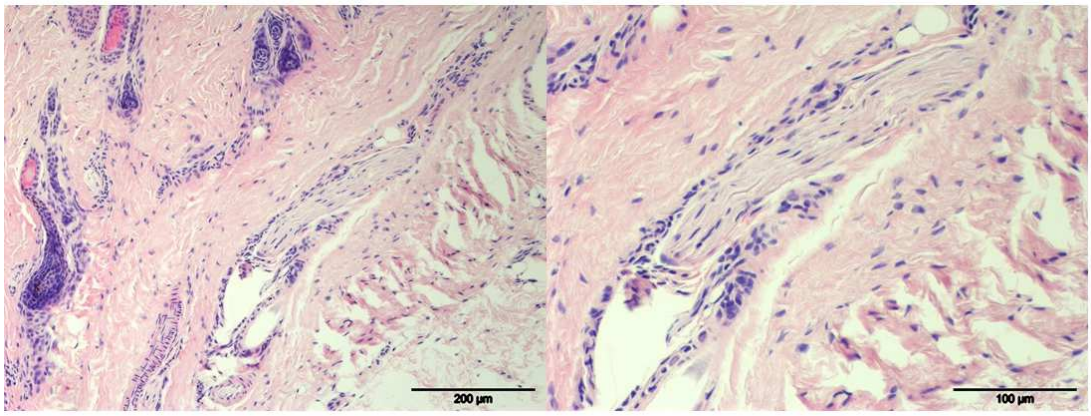


FIGURE 59: MINIMAL PERINEURAL INFLAMMATORY INFILTRATE OBSERVED IN A SECTION OF THE HOCK SKIN OF ARC016_17. THIS COULD POTENTIALLY DEVELOP INTO A LEPROSY LESION (BIN IN ZN STAIN = 0)

The Ridley-Jopling categories assigned in the remaining samples are detailed in Table 18. Figure 60 through to Figure 63 show examples of lesions representative of the different categories.

TABLE 18: RIDLEY-JOPLING CATEGORISATION OF LESIONS SEEN IN THIS SET OF ERS SAMPLES: NUMBER OF ERS PLACED IN EACH CATEGORY AND BIN'S ASSIGNED (NO. OF ERS A PARTICULAR BIN WAS ASSIGNED TO; FOR BIN CATEGORY DEFINITIONS SEE P. 10 ET SEQ.)

Tissue	Frequency with which lesions of each category (InL, BB, BL, LL) were observed and bacterial indices (BIn) seen							
	InL	BIn	BB	BIn	BL	BIn	LL	BIn
Ear	4	0 (n=1) 1 (n=2) 6 (n=1)	0	NA	1	6 (n=1)	5	5 (n=1) 6 (n=4)
Hock skin	1	0 (n=1)	2	4 (n=1) 6 (n=1)	0	NA	4	4 (n=1) 5 (n=1) 6 (n=2)
Nose	0	NA	0	NA	2	4 (n=2)	4	4 (n=2) 5 (n=1) 6 (n=1)
Scrotal skin	0	NA	0	NA	0	NA	1	5 (n=1)

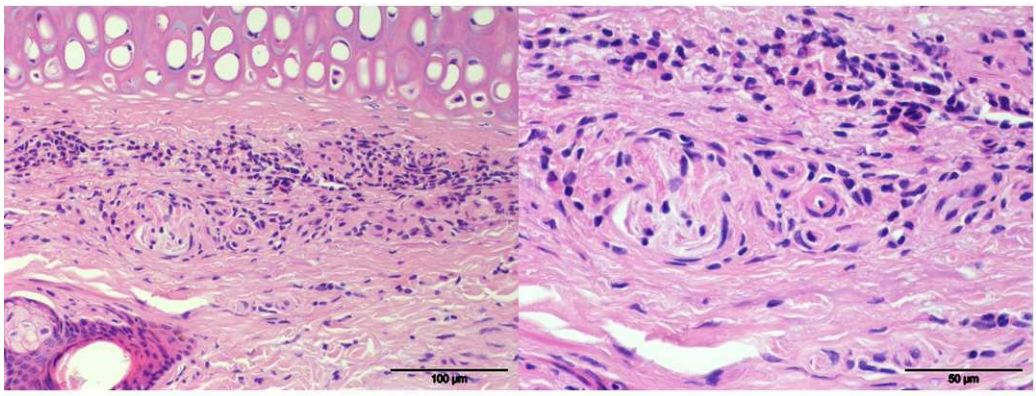


FIGURE 60: EXAMPLE OF THE INFLAMMATORY REACTION SEEN IN THE EAR LESION OF BIC006_16, CLASSED AS INL (BIN IN ZN STAIN = 1).

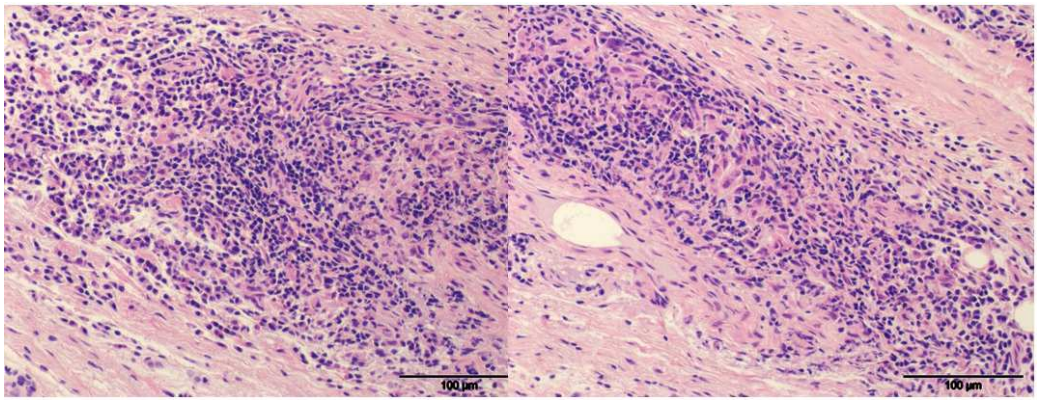


FIGURE 61: EXAMPLE OF THE INFLAMMATORY REACTION SEEN IN THE HOCK LESION OF BIC001_16, CLASSED AS BB (BIN IN ZN STAIN = 4)

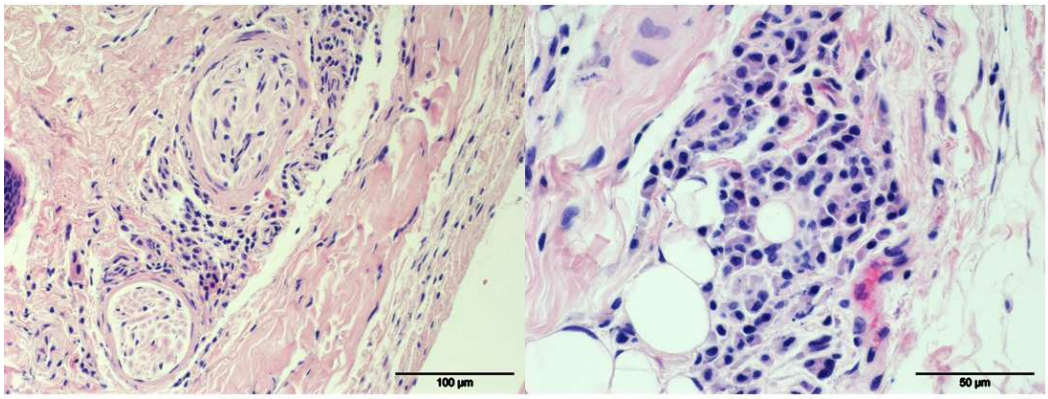


FIGURE 62: EXAMPLE OF INFLAMMATORY INFILTRATE OBSERVED IN NASAL SKIN OF BIC016_17 AND CLASSED AS BL (BIN IN ZN STAIN = 4)

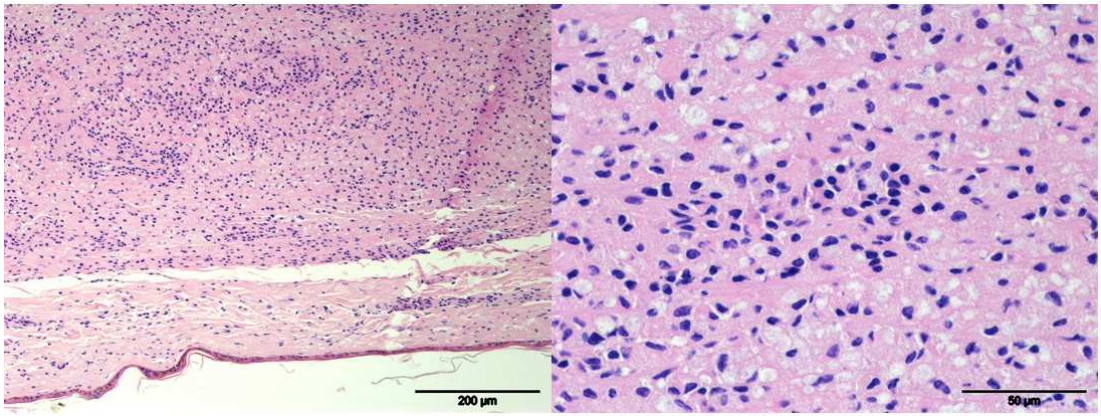


FIGURE 63: EXAMPLE OF INFLAMMATORY INFILTRATE OBSERVED IN THE EAR OF BIC003_16 AND CLASSED AS LL (BIN IN ZN STAIN = 6)

In one of the LL lesions seen in hock skin, characteristics were observed that may indicate an ENL or a secondary infection (Figure 64). There were acute inflammation and necrosis reaching the epidermis and lymphocytes and epithelioid cells observed in the deep dermis.

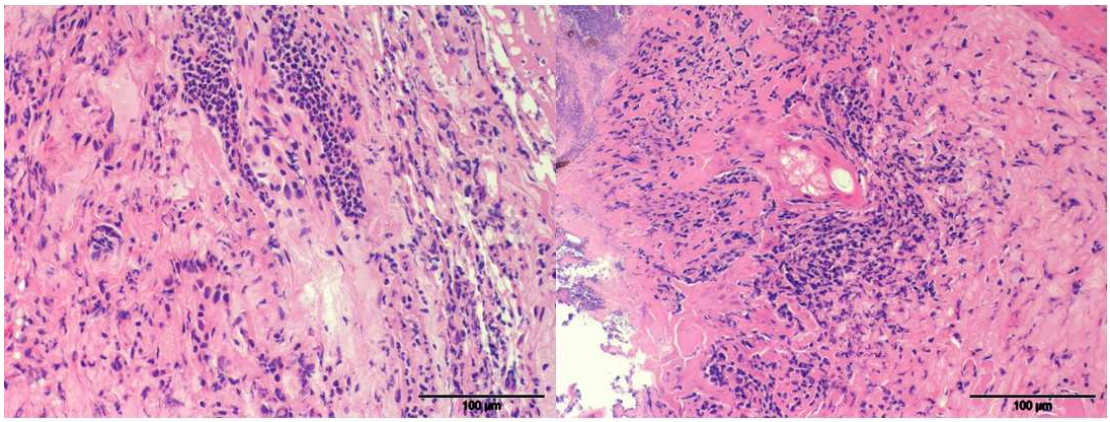


FIGURE 64: RANGE OF INFLAMMATORY INFILTRATES OBSERVED IN THE HOCK OF BIC007_16. LEFT: LYMPHOCYTES AND EPITHELIOID CELLS IN THE DEEP DERMIS; RIGHT: ACUTE INFLAMMATION AND NECROSIS REACHING THE EPIDERMIS

No presentations consistent with a borderline tuberculoid or tuberculoid form of leprosy were present in any of the samples. Even with expert advice the classification of the lesions was not always straightforward.

There is no difference in the ability to identify ERS leprosy cases using molecular and histopathological methods

Only tissues that were assessed with both molecular and histological methods were included in this comparison, i.e. 10 for all eleven carcasses (ear, eye, hock skin, nose, muzzle, front footpad, hind footpad, lung, intestines, spleen, liver, and kidney; n= 110), and one for 10 carcasses (lung). Thus, a total of 120 tissue samples from ERS affected by leprosy were used to assess whether molecular and histopathological methods would return positive results equally. Eyes and mandibular lymph nodes were excluded as the numbers available for histological and molecular assessment differed. Mamma and testis were excluded as

they were only assessed histologically and here found to be negative in all instances as well as being collected sex specifically.

Molecular methods were able to confirm the presence of leprosy bacilli in more tissues than histological methods. Not all tissues tested positive with either method, despite all carcasses being affected by leprosy. No *M. leprae* DNA or AFB were detected in 49.2% ($n_{Neither}$= 59) of the tissue samples examined. Out of the 61 tissue samples in which one or the other was detected 49.2% were identified with both methods, 47.5% only by PCR and 3.3% only using histological methods (Table 19).

The null hypothesis that whichever test is used does not affect the outcome, i.e. whether a tissue is identified as containing leprosy bacilli, needs to be rejected (Fisher's exact test, p= 0.0000000000152). Molecular methods are able to identify the presence of leprosy more often than histological methods. One squirrel (ARC016_18) would have been completely missed as being affected by leprosy with histological methods alone.

TABLE 19: NUMBER OF TISSUES FROM LEPROUS SQUIRRELS THAT TESTED POSITIVE FOR *M. LEPRAE* DNA, THE PRESENCE OF AFB OR BOTH.

	Test with positive result			
M. leprae DNA and/ or AFB detected	PCR	Both	AFB	Neither
	29	30	2	59

There is no difference in the ability to identify ERS leprosy cases using a range of different tissues

Up to 13 different tissues/organs per carcass were assessed using molecular methods and up to 14 were assessed macroscopically and histologically. Macroscopic lesions were only present in a small number of tissues per carcass, or not at all. In no animal were all assessed tissues positive. In ERS with severe lesions more than half of the assessed tissues contained *M. leprae* DNA and more than 14% of the tissues contained AFB. In ERS without or with only mild clinical lesions *M. leprae* DNA and AFB could be isolated from a much smaller proportion of organs and AFB were present in less than a sixth of the assessed tissues (Table 20).

Pathognomonic macroscopic skin lesions were seen on hocks (7 out of 11 carcasses (63.6%)), ears (6 out of 11 carcasses (54.5%)), and noses (3 out of 11 carcasses (27.3%)). Another form of macroscopic lesion that was deemed as potentially due to a leprosy infection was a swelling of the mandibular lymph nodes (2 out of 9 carcasses (22.2%)). No macroscopic changes that would have been assigned to leprosy were seen in any of the other tissues. In all macroscopically identified leprosy lesions AFB and *M. leprae* DNA were present. However, far more tissues contained leprosy bacilli than one would have expected based on the macroscopic lesions. Thus, while macroscopic lesions can be helpful in

choosing a sampling site for leprosy diagnostics, detecting the presence of leprosy bacilli is possible without them.

TABLE 20: SUMMARY OF TISSUES AVAILABLE FOR MACROSCOPIC, MOLECULAR AND HISTOLOGICAL ASSESSMENT FROM THE 11 CARCASSES INCLUDED IN THIS ANALYSIS, ALONG WITH THEIR LEPROSY SEVERITY CATEGORY, AND THE PROPORTION OF POSITIVE SAMPLES IN EACH ASSESSMENT TYPE.

Animal ID	No of tissues macroscopic assessment	No of tissues with obvious lesions (%)	Leprosy category	No of tissues for PCR	No of tissues positive (%)	No of tissues for histology	No of tissues with AFB (%)
BIC001_16	14	4 (28.6)	4	13	11 (84.6)	14	5 (35.7)
BIC002_16	14	4 (28.6)	4	13	11 (84.6)	14	7 (50.0)
BIC003_16	14	2 (14.3)	4	13	8 (61.5)	14	6 (42.9)
BIC007_16	13	2 (15.4)	4	13	11 (84.6)	13	5 (38.5)
BIC014_17	14	3 (21.4)	4	13	10 (76.9)	14	5 (35.7)
BIC016_17	14	2 (14.3)	4	13	7 (53.8)	14	2 (14.3)
BIC010_16	13	1 (7.7)	1	13	5 (38.5)	13	2 (15.4)
BIC006_16	13	0 (0)	0	12	1 (8.3)	13	1 (7.7)
BIC009_16	14	0 (0)	0	13	1 (7.7)	14	1 (7.1)
BIC018_18	11	0 (0)	0	10	2 (20)	11	1 (9.0)
ARC016_18	13	0 (0)	0	13	1 (7.7)	13	0 (0)

Looking in more detail into the tissues from which *M. leprae* DNA was isolated, ears and spleen are those from which it was most frequently possible to isolate bacterial DNA, followed by the liver and hock skin (Figure 65, p. 130). In the other organs bacterial DNA was detected less frequently and mostly in severely affected squirrels.

In the histological assessment the ear was the tissue in which AFB were most frequently seen again, followed by the spleen, nose, hock, mandibular lymph nodes and the liver (Figure 66, p. 130). Generally, the paler colours implied that it was less frequently possible to identify AFB using histological methods than it was possible to isolate bacterial DNA.

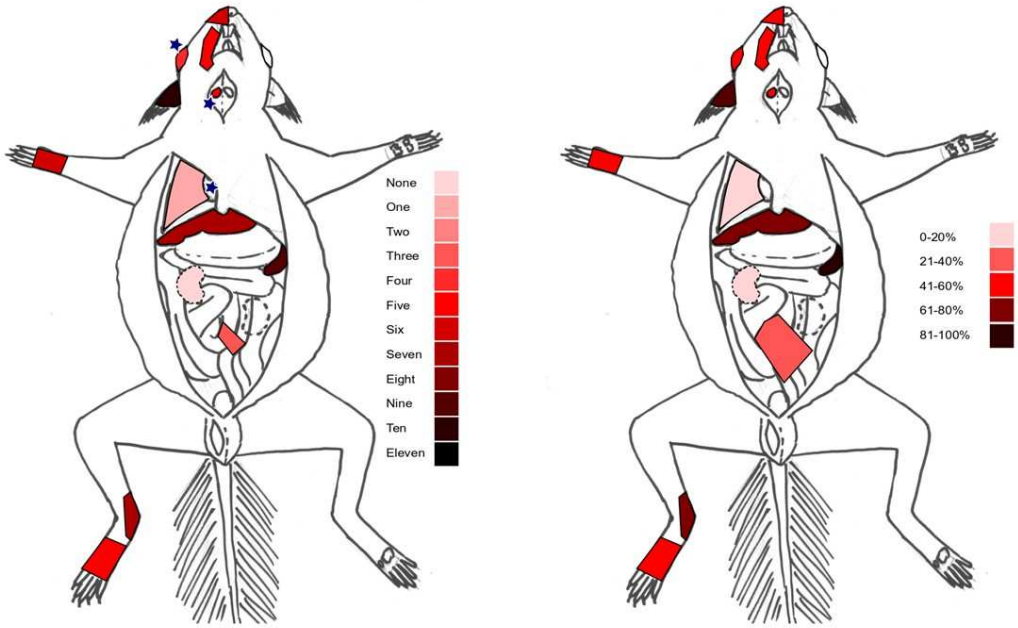


FIGURE 65: *M. LEPRAE* DNA ISOLATION FROM LEPROSY AFFECTED ERS CARCASSES: COLOUR RANGE REPRESENTS THE NUMBER (LEFT, BLUE STARS MARKING TISSUES NOT AVAILABLE FOR ALL CARCASSES) OR PROPORTION (RIGHT, RELATIVE TO THE ACTUAL NUMBER OF SAMPLES AVAILABLE PER TISSUE) OF CARCASSES IN WHICH ISOLATION WAS ACHIEVED FROM A PARTICULAR TISSUE

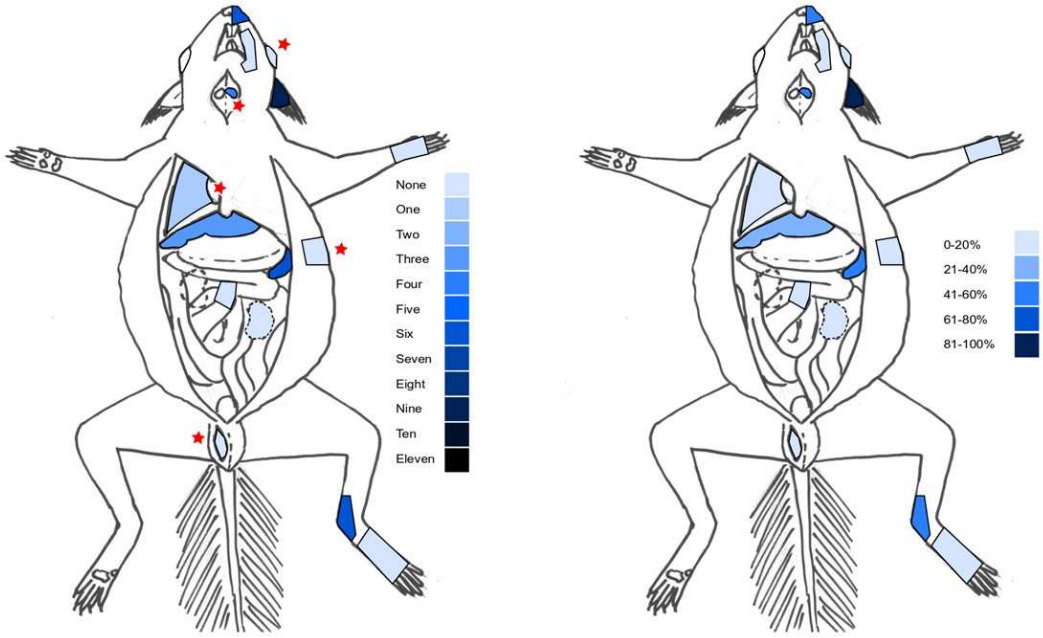


FIGURE 66: AFB IN ERS CARCASS TISSUES: COLOUR RANGE REPRESENTING THE NUMBER (LEFT, RED STARS MARKING TISSUES NOT AVAILABLE FOR ALL CARCASSES) OR PROPORTION (RIGHT, RELATIVE TO THE ACTUAL NUMBER OF SAMPLES AVAILABLE PER TISSUE) OF ERS CARCASSES WITH AFB IN A PARTICULAR TISSUE

Often the presence of leprosy bacilli in a particular tissue could be detected with both molecular and histological methods. In three instances in three separate tissues histological methods were able to identify the presence of leprosy bacilli in a tissue that would have been missed using molecular methods, while molecular methods detected their presence in 35 tissues where no AFB were seen. Only combining both methods in the ear allowed the identification of all 11 squirrels as affected by leprosy. Using any other tissue, some affected ERS would have been missed. In front and hind footpads, muzzle, eye, and intestines only *M. leprae* DNA was identified but in no instance AFBs. Kidneys are not a suitable tissue to use in leprosy diagnostics in ERS, as they did not contain bacteria in any of the carcasses assessed (Table 21).

TABLE 21: PROPORTION OF ERS THAT WOULD HAVE BEEN IDENTIFIED AS AFFECTED BY LEPROSY IF ONLY ONE TISSUE HAD BEEN USED AND METHODS THAT LET TO THESE POSITIVE RESULTS

Tissue	Proportion of squirrels diagnosed	Test with which the presence of *M. leprae* was detected			
		PCR	Both	AFB	Neither
Ear	100 %	2	8	1	0
Spleen	81.1 %	3	6	0	2
Liver	63.6 %	4	3	0	4
Hock skin	63.6 %	0	7	0	4
Mandibular lymph node*	60 %	2	3	1	4
Nose	54.5 %	0	6	0	5
Front footpad	54.5 %	6	0	0	5
Hind footpad	45.5 %	5	0	0	6
Muzzle	45.5 %	5	0	0	6
Eye*	40 %	4	0	0	6
Intestines	27.3 %	3	0	0	8
Lung*	20 %	1	0	1	8
Kidney	0 %	0	0	0	11
Mamma#	0 %	NA	NA	0	7
Testicle#	0 %	NA	NA	0	4

**Tissues were not available for all carcasses and/or not for both tests*
#Tissues were only available depending on squirrel sex and only assessed histologically

In ERS carcasses severely affected by leprosy, *M. leprae* DNA was present across a range of 12 tissues, and AFB were detected in seven different tissues. The bacteria were therefore widely distributed through the ERS's body. Even in non-clinically/mildly affected squirrels bacterial DNA could be detected in the skin (ear, hock) internal organs (spleen, liver) and in the eye. Histological detection of AFB in this group was difficult and only possible in ear (BIn= 1, 1, and 6, respectively), and in one instance each in the spleen (BIn= 1) and hock (BIn= 6).

A high BIn makes it likely that the bacteria were actively reproducing in a tissue, while a low BIn could also occur in tissues that do not offer the bacteria optimal conditions for growth. The highest BIn's were observed in skin tissue, the ear clearly being the one tissue with the

highest bacterial loads followed by the hock and nose. Bacterial loads in the lymph nodes were more variable and may not only be influenced by bacterial growth in the lymphatic tissue but also by bacteria from the head area being filtered and retained there. AFB were less frequently detected in internal organs than in skin tissues and the BIn was lower (Table 22, Figure 67).

TABLE 22: BIN NOTED FOR TISSUES OF CARCASSES SEVERELY AFFECTED BY LEPROSY IN WHICH AFB WERE DETECTED

Tissue	BIn's observed in the tissue across the carcasses					
Ear (n= 6)	6	6	6	6	6	5
Hock skin (n= 5)	6	6	5	4	4	-
Nose (n= 6)	6	5	4	4	4	4
Mandibular lymph node (n= 4)	6	5	4	1	-	-
Spleen (n= 5)	4	2	1	1	1	-
Liver (n= 3)	4	1	1	-	-	-
Lung (n= 1)	1	-	-	-	-	-

FIGURE 67: NUMBER OF TIMES IN WHICH A BIN COULD BE DETERMINED IN A TISSUE (LEFT) AND MEDIAN BIN OBSERVED IN THESE TISSUES (RIGHT).

4.4. Discussion

Data presented in chapter 3 implied that leprosy signs in ERS are more variable than previously reported looking only at carcasses (Meredith *et al.*, 2014; Avanzi *et al.*, 2016). The data introduced in this chapter supports this notion, both with respect to clinical signs of leprosy, which are found to vary between individuals while sharing some common characteristics and with respect to histological presentations where InL and BB lesions were

described for the first time. The data also showed a minor impact of leprosy on individual ERS health (assessed using three health indicators) and that ERS sex does not influence disease presentation. The remaining aims of this chapter were also successfully achieved by demonstrating that molecular methods are more likely to detect the presence of leprosy bacilli DNA in an ERS than histological methods are to detect AFB and establishing that the ear is the optimal sampling location for ERS leprosy diagnostics. These results are discussed in detail below.

4.4.1. Clinical presentation of leprosy in ERS

In terms of new information on the clinical presentation of leprosy in ERS, only one juvenile ERS colonised by *M. leprae* could be included in this study. The clinical signs discussed here are thus reflecting leprosy in adult ERS.

The proportion of ERS seen with clinical signs of disease in this study is also worth considering. While 55 ERS were identified as affected by leprosy, only 34 had clinical signs of the disease. Thus, just 38.2% of ERS affected by leprosy bacilli did not (yet) show clinical signs of disease. While clinical disease thus appears to be more frequently observable in ERS than in for example NBA (Truman, 2005; Morgan and Loughry, 2009), this observation made in the context of this particular study should not let one jump to generalised conclusions. All clinically diseased ERS came from the BI population, none from the AR population, even though colonisation with *M. leprae* was detected here. Clinical cases previously identified in surveillance efforts in Scotland were rare (Meredith *et al.*, 2014). In other host species only a small proportion of individuals is thought to be likely to develop clinical leprosy following natural infection (5-10% in humans (Scollard, Truman and Ebenezer, 2015), 5% in NBA (Truman *et al.*, 1986)). It is therefore conceivable that the situation in the wider British ERS population is actually similar to that in other hosts, with few individuals developing clinical disease. This reinforces the notion that it is important to reliably identify ERS colonised and sub-clinically infected by leprosy bacilli, if the true epidemiological situation in this host species is to be evaluated. This is addressed further in chapter 6 (p. 169 et seqq.). It also highlights the need for targeted future studies to be conducted in other ERS populations to assess the average risk for a British ERS infected with leprosy bacilli to develop clinical signs of disease.

Leprosy lesions are pathognomonic. They are very similar between individuals.

ERS clinically identified as leprosy suspects were confirmed to be infected with *M. leprae* in almost all instances (91.2%) in this study. It cannot be said whether this proportion would have been higher, if PCR data had been available for all ERS. The sensitivity of the αPGL-I UCP LFA is 88%, so a false negative serology in these two instances is possible. In the one ERS (BI100_18) clinically identified as leprosy suspect in which both PCR and serology were negative, the lesion was very small (lesion score 4, lesion category 1) and the tissue sample

for PCR collected from the unaffected ear. Thus, false negative laboratory results are possible, and retesting at a later point in time or PCR analysis of lesion tissue might have confirmed this ERS as leprosy case as well. Overall, the definition used for clinical lesions (p. 89) in this study thus appears to be a good guide for the identification of leprosy cases by an experienced assessor.

The two surveillance cases (R3-17, R38-18) that were not leprosy cases, despite initial clinical suspicion, did show distinct differences in their clinical presentation. While the ear was beginning to thicken and showed incomplete hair loss in R3-17, the lesions on chin and eyelids were larger and more advanced than the ear lesions. In the leprosy cases seen in this study the ear and hocks were the primary location of leprosy lesions, other body areas only becoming affected in advanced stages of the disease. All lesions on R3-17 presented with some fur cover, making them quite distinct from leprosy lesions of the same size described so far. The cut surface of the lesions in R3-17 was pale and not bulging and thus different from what has been observed in leprosy lesions. Such lesions could however easily be mistaken for leprosy lesions by an examiner with limited experience or during a superficial assessment by an assessor with ERS leprosy experience.

For R38-18 the hair loss and thickening of the skin was limited to the nose, the ears and hocks were unaffected and the ERS was still very young. These differences in lesion pattern should be considered before the suspicion of leprosy is proposed. The low number of ERS identified as leprosy suspects in the squirrel surveillance scheme during the two years of this study may again indicate how rare clinical leprosy is in the wider Scottish ERS population. It could also indicate that the currently published descriptions allow those familiar with them to identify most non-leprous ERS as such.

Looking at the description of other ERS skin diseases in the literature malignant melanomas are very rarely described and in the case report again had a different distribution pattern from leprosy lesions, occurring first on the eyelids (Fukui *et al.*, 2002). In all other skin infections a more scaly, exudative or purulent presentation than what is typical for leprosy can be expected (McInnes *et al.*, 2009; Simpson *et al.*, 2013; Wibbelt *et al.*, 2017). Therefore, it appears appropriate to say that ERS leprosy lesions are pathognomonic. This could however change, as more skin conditions keep being described for ERS. In humans, where the body of dermatological conditions described is far more extensive, even every form of leprosy has its own range of differential diagnosis (Moschella and Garcia-Albea, 2016).

Observations in autumn 2016 (chapter 3, p. 80) had suggested that leprosy lesions form on ears and hock first. Data presented in this chapter supports this initial observation, and adds the scrotum in male ERS to the list of likely locations for early leprosy lesions. Unlike in humans, no leprosy lesions have been observed on the torso of ERS.

Clinical presentations of leprosy in ERS, while similar in location and texture of the lesions, are variable when it comes to the size of lesions, their shape, particularly in advanced cases, and the presence and absence of ulcerations. ERS leprosy lesions seen in this study were mostly categorised as mild or severe and only seven were placed in categories 2 and 3 (20.6%). Longitudinal datasets, such as presented in chapter 5, could address whether categories 2 and 3 represent transient stages of the infection and could be summarised into one category to simplify the system established in this study going forward.

In humans and NBA individual variability of clinical lesions is high, allowing for five main forms of clinical presentation to be defined. However, the lesions seen here and currently described for ERS vary on a narrower spectrum. Single nodular lesions, as they appear in ERS leprosy cases are described for LL in NBA as well (Frota *et al.*, 2012; Sharma *et al.*, 2013). Multiple, but not single, nodules with a shiny surface are described in human BL leprosy. However, in this species hair loss in this form is usually incomplete (Bhat and Prakash, 2012). Based on the descriptions provided by Simpson *et al.* (2015) the ERS leprosy lesions observed on the Isle of Wight are similar to some InL in humans (Bhat and Prakash, 2012). Still, while there are similarities in the clinical presentation of leprosy in the three most intensely researched hosts, differences do exist, and it should be avoided to try and categorise pathognomonic clinical presentations of leprosy in ERS using the system developed for humans. Where they are similar, similarities can be pointed out, but at this early stage in ERS leprosy research it should be considered that host-pathogen interaction determining the clinical presentation resulting from an infection with leprosy bacilli may differ between the species.

Leprosy has an impact on ERS beyond the immediate clinical signs

Being colonised or clinically infected with *M. leprae* does not have a statistically significant effect on BCS or GHS in ERS. Severe lesions may however have a negative impact that while not statistically significant, may be biologically relevant for the individual.

ERS showing clinical signs of leprosy were found to be significantly heavier than colonised or unaffected ERS. Within the group of clinically diseased ERS weight showed a slight tendency to increase with lesion severity category, though this correlation was not statistically significant. A similar pattern has been described in NBA (Truman *et al.*, 1991). As in NBA the higher weight could imply that these ERS are older than the ERS in the other groups, though weight gain in adulthood has not been studied specifically in ERS. As it is notoriously difficult to age ERS once they are adult (Bosch and Lurz, 2012), further research based on this observation could offer relevant information not just for ERS leprosy research but for ERS research in an ecological context, where for example the average age of members of the population could be of interest.

Influence of ERS sex on leprosy

In humans, leprosy is not a sex specific disease, while in NBAs one study suggested that females may be more frequently affected, while others did not find an effect of sex on leprosy susceptibility in this species (Scollard *et al.*, 2006; Morgan and Loughry, 2009). In ERS sex and leprosy susceptibility do not appear to be linked based on the sample set available in this study. Lesion severity does also not appear to be linked to ERS sex. However, in humans, certain events that only occur in females, such as pregnancy and the associated hormonal changes, have been reported to be risk factors in LL patients for developing ENL (White and Franco-Paredes, 2015). It could therefore be of interest to particularly monitor female ERS with clinical signs of leprosy using a hands-off approach like monitoring a maternal drey with camera traps during the breeding period to assess whether pregnancy has an effect on their disease intensity or is linked to complications.

A limitation of this study is, that no ERS infected with *M. lepromatosis* were available for assessment. Skin lesions caused by *M. leprae* and *M. lepromatosis* previously have been described to be similar (Avanzi *et al.*, 2016). However, on the Isle of Wight, crusty lesions have been described in ERS infected with *M. lepromatosis*, which have not been described elsewhere (Simpson *et al.*, 2015). Thus, while some of the information collected in this chapter on leprosy presentation in ERS infected with *M. leprae* may well be transferrable, it remains possible, that an infection with *M. lepromatosis* can cause additional presentations that the current work did not cover.

In summary, the data presented in this chapter has fulfilled the aim of providing new information of the characteristics of clinical leprosy in ERS. It has underlined that leprosy lesions in ERS appear to be pathognomonic, and flagged up, that while they do look similar to lesions described in other host species, they are distinct in some respects, and appear slightly less variable. They should at this early stage of ERS leprosy research be assessed with an open mind. It should be avoided to try to fit them into categories defined for other hosts. Leprosy has little impact on the general health of affected individuals and does not appear to be a sex-specific disease in ERS. It may be a disease of older ERS.

4.4.2. Histopathological and molecular assessment of leprosy affected ERS

While not all histological presentations of leprosy described in other hosts were seen in this study, i.e. the fourth hypothesis of this chapter was not confirmed, some new histological presentations of ERS leprosy lesions were observed. They are discussed below, particularly considering why it would be relevant to further try and determine the full extent of histological leprosy presentations in ERS. In respect to the fifth hypothesis, it was possible to establish in this study that molecular methods are more often able to identify leprosy cases than histological methods. The study also showed that specific tissues, particularly the ear and spleen, can increase the chances of identifying ERS colonised by or infected with leprosy

bacilli. In severe clinical leprosy cases bacteria are present in a wider range of tissues than in mild cases or colonised ERS. Thus, the sixth hypothesis of this chapter had to be rejected.

The whole histological spectrum of leprosy lesions is present in ERS

It was possible to describe inflammatory reactions showing the characteristics of two additional histological categories of leprosy in ERS in this study (InL and BB) in addition to the ones previously published (LL, BL and BT) (Avanzi *et al.*, 2016). All lesions seen in this study were InL, BB, BL or LL, thus further strengthening the impression that lesions on the lepromatous end of the spectrum are much more likely to be detected in ERS than tuberculoid lesions, with TT lesions remaining undescribed. However, to establish whether the inflammatory reactions seen in ERS resemble those in the other host species or are indeed fully comparable, a specialised leprosy histopathologist should compare sections from across the host spectrum directly with each other. It was very difficult to class ERS slides based on published descriptions of the other two hosts. It is possible that ERS inflammatory reactions do not perfectly fit the Ridley-Jopling categorisation system. To learn more about the CD4+/CD8+ ratio in ERS leprosy lesions immunohistochemical staining should be employed, as this would offer additional information as to where on the Ridley-Jopling scale lesions of this species fit in.

The prominent nerve invasions with large numbers of AFB described for other hosts (Han *et al.*, 2008) has not been observed in ERS yet, while some nerve involvement, particularly in the form of lymphocyte cuffs or individual AFB in nerves have been seen. Within clinically visible granulomas in ERS normal structures like nerve bundles or larger vessels were no longer identifiable. Granulomas in ERS appeared to often form initially close to the cartilage and then expanded through the dermis until they reached the epidermis and, in some instances, ulcerated. This pattern is very different from what is described for BL-LL lesions in humans and actually in some respects similar to what is described for TT lesions in humans (Ridley and Jopling, 1966; Massone, Belachew and Schettini, 2015). However, other characteristics, like globi formation and large numbers of foamy macrophages have placed ERS leprosy lesions at the lepromatous end of the histological spectrum. The Ridley-Jopling classification system was originally introduced for research purposes to reflect to which extent a patient is resistant to/able to produce an adequate immune response to an infection with leprosy bacilli (Ridley and Jopling, 1966). With this original goal in mind, it currently appears worthwhile to detail cell types and involvement of different structures when describing the histology of ERS leprosy lesions instead of placing lesions into a Ridley Jopling category. The latter could miss important, unique characteristics in the immune response ERS show to an infection with leprosy bacilli.

There is no difference in the ability to identify ERS leprosy cases using molecular and histopathological methods

Molecular methods in this study used a cubical tissue section, while the histopathological sections are ultrathin, but can in the case of an ERS, span a complete organ. In both methods, leprosy bacilli could be missed, if they are very localised. Still, molecular methods were more sensitive in detecting bacterial presence in this study.

In humans Fite-Faro (FF) is the accepted standard stain for AFB detection in leprosy patients and described as more sensitive than Ziehl-Neelson (ZN) staining in humans and NBA (Walsh, Meyers and Binford, 1986; Scollard, Truman and Ebenezer, 2015). It is thought to be the more reliable stain as *M. leprae* is sensitive to an additional alcohol decolorizing step occurring in ZN staining compared to FF and may thus show less well (Rendini and Levis, 2017). In ERS diagnostic success using ZN staining has been good, and the laboratory used in this study had experience in this staining method. Future research could assess whether a modification of the staining process would offer advantages in ERS too, or if there is no significant difference between the two staining techniques in this species. If FF were to perform significantly better than ZN this could close, or at least narrow, the gap between molecular and histological methods.

Both methods can complement each other, depending on the situations in which a leprosy diagnosis is needed. Molecular methods are fast and can be automated for high throughput. They are thus highly suitable for pre-translocation disease screening and surveillance. Producing and analysing histological slides of the same number of ERS can take much longer, depending on investigator speed and experience. However, for a low number of samples bacteriological staining can be performed within hours to assess whether a tissue contains AFB. Thus, if the aim is to quickly establish whether a single ERS is a MB leprosy case, using histological methods could be an advantage. However, acid-fast stains are not species specific and would only be diagnostic for leprosy if AFB are seen within a nerve (Massone, Belachew and Schettini, 2015), while the PCR is highly specific. The PCR protocol used in this study is producing a qualitative, not a quantitative result. Therefore, if it is of interest how many leprosy bacilli are present in a tissue, it needs to be backed up with histological methods that allow a BIn to be established. Alternatively, and likely to be more accurate, a quantitative real time PCR for the detection of *M. leprae* DNA, as already recommended for use in humans (Braet *et al.*, 2018), could be applied to ERS. Only histological methods currently allow a characterisation of the cells involved in the formation of a leprosy lesion and thus offer information on the host reaction to the pathogen.

Neither method, as used in this study, readily allows to determine the viability of leprosy bacilli identified. This is a gap that will be difficult to address, based on the nature of most ERS samples available for full post mortem assessment, which often present in advanced stages of decay. However, reverse transcriptase PCR to detect *M. leprae* RNA could show

the presence of viable *M. leprae* (Turankar *et al.*, 2016), if the technique was adapted for the use in ERS samples. Another option to explore in future research efforts to determine the viability of *M. leprae* detected in ERS tissues could be the adaption of bacteriophage-based methods combined with PCR, which are currently established for *Mycobacterium avium* subsp. *paratuberculosis* and for *Mycobacterium bovis* (Swift, Convery and Rees, 2016). This method may not be adaptable due to the specific characteristics of *M. leprae*, but if it was successfully adapted, it could allow for a highly informative diagnosis within 48 hours (Swift, Convery and Rees, 2016).

There is no difference in the ability to identify ERS leprosy cases using a range of different tissues

Leprosy bacilli were detected in a range of tissues. This implies that while macroscopic leprosy lesions in squirrels are mostly restricted to the skin, the distribution of the bacteria is not limited to the periphery, even in non-clinical and mild cases. This was unexpected, given the relatively high core body temperature of ERS (38-40°C) (Bosch and Lurz, 2012). However, no AFB were detected in ERS testes and kidneys, the latter also being PCR negative, organs that have been described as containing AFB in humans with LL leprosy (Soares *et al.*, 2017). It could be possible that these organs would become infected, if the disease had had more time to progress, as for NBA involvement of all organs is described as well, but only after extended periods of time (Truman, 2005). No study comparable to the one presented here to establish the optimal tissue for leprosy diagnosis has been published for NBA or humans.

Of the skin tissues assessed in this study, footpads, both front and back, contained *M. leprae* DNA in only about half of the cases and in no case were AFB or inflammation seen histologically. The whole chest/front leg area appears to be largely unaffected by leprosy in ERS. On the hind legs it is important to notice that the hock rather than the foot is the initial location for leprosy lesions, even though lesions can extend towards the foot in advanced stages.

Interestingly, AFB were only observed in the nose in severe leprosy cases and not in mild ones or in colonised ERS. The transmission of leprosy is still not fully understood, however nasal discharge, which can contain large numbers of bacteria in humans, is thought to play a role (Bratschi *et al.*, 2015). In ERS bacteria only appear to be present here late in the disease history, and profuse discharge from the nose is unusual in ERS. Thus, other mechanisms may be more relevant for disease transmission in this species.

Lower bacterial loads in internal organs (Figure 67, p. 132) could imply that bacteria are not as readily multiplying in these warmer body areas as they are in the ear (median BIn 6) or hock (median BIn 5). A similar pattern is described for NBA (Frota *et al.*, 2012).

Conclusion

Clinical lesions caused by *M. leprae* in ERS appear to be pathognomonic, as they all share some uniting characteristics that are not found in other skin conditions currently described in ERS. Leprosy, at least in cases that are less than severe, does not seem to have a negative impact on general ERS health. It may be a disease of older ERS. Leprosy in ERS presents in some features similar to leprosy in other host species, but neither clinically nor histologically does it perfectly match the criteria for the individual categories established for humans. At this early stage of ERS leprosy research it is thus useful to invest time in more detailed and species-specific documentation systems to explore the full spectrum of lesions present in this host. Direct comparisons of samples from the different host species by specialists should be made to determine to what extent categories established in humans are really applicable to this new host. Exploring the similarities and differences further will be of interest to determine whether lessons from human medicine can be used to understand host-pathogen interactions in ERS or if they need to be considered with a pinch of salt. Molecular methods were able to detect *M. leprae* in more individuals and tissue types than histological methods. The ear is the best location for diagnostic leprosy sampling in ERS. Not only is it the one location in which bacteria are found in most affected ERS, it is also a location in which high BIn are regularly observed in this host.

Chapter 5: Disease progression of leprosy in ERS

5.1. Introduction

As pointed out in previous chapters leprosy was initially described in ERS carcasses. This did not allow any inference on incubation period or disease progression in this species (Meredith *et al.*, 2014; Simpson *et al.*, 2015; Avanzi *et al.*, 2016; Butler *et al.*, 2017). ERS are too dissimilar in their life history and biology from other host species such as humans and NBA to assume outright that information available about leprosy development and progression in these species can be transferred to ERS.

Rapid development of leprosy lesions (2 months) in humans is an exception. On average two to four years pass between likely time of infection and the development of clinical disease. Sometimes it takes decades (Lastória and de Abreu, 2014b). The intensification of symptoms is also slow, implied by the fact that even after medical attention is sought, a correct diagnosis is sometimes not made for years (Li *et al.*, 2016). In wild NBA clinical leprosy is rare and is usually observed in NBA older than two years, which led to an estimate for the incubation period in this species of 12-24 months (Walsh, Meyers and Binford, 1986; Oli *et al.*, 2017). Even under laboratory conditions and high infection pressure the development of initial clinical signs takes three months or up to a year (Sharma *et al.*, 2018). In the two most studied hosts, leprosy clearly presents as a very slowly progressing disease.

While Avanzi *et al.* (2016) described the presence of leprosy bacilli DNA in young ERS, they only observed clinical signs of disease in adults, i.e. ERS that were likely to be older than 9 months. The aim of this part of the study was to determine whether the development and progression of clinical leprosy lesions could be observed in ERS within a two-year timeframe. The detailed clinical assessments of leprosy and health status made at each field assessment and results of molecular and serological tests were utilised to investigate lesion progression in individual ERS and provide a first, cautionary estimate of the incubation period in this species.

5.2. Methods

ERS assessments, sampling and sample analysis were carried out as described in chapters 2 and 3. All ERS trapped and released in the six-monthly sampling sessions on BI and AR were microchipped. Scanning for the presence of a microchip was always performed at the end of the data collection to avoid unconscious bias in the assessment of returning animals.

Information on the presence/absence of leprosy lesions, the lesion category, lesion score, results of serological tests, where applicable PCR, and the resulting diagnosis reached (Figure 37, p. 99), were considered in conjunction with age, sex, breeding condition, BCS, weight and GHS of each ERS.

In this chapter results are presented as a timeline, starting from an animal's initial assessment (time point 0) in six months steps, up to 24 months. Thus, animals that were seen for example in session 3 for the first time could not possibly provide a full dataset, but maximally provide data for 0, six and 12 months. The reader can determine in which session an ERS was assessed first from the animal ID, as summarised in Table 23.

TABLE 23: SAMPLING SESSIONS AND THE RANGE OF SHORT ANIMAL ID'S ASSIGNED, IF AN ERS WAS FIRST SEEN IN EACH

	Animal ID's for which initial assessment occurred	
Session	BI	AR
1	BI 1 to BI 26	NA
2	BI 27 to BI 52	AR 1 to AR 17
3	BI 53 to BI 72	AR 18 to AR 23
4	BI 73 to BI 97	AR 24 to AR 37
5	BI 98 to BI 126	AR 38 to AR 62

To understand the timescales on which leprosy acts in ERS it is important to have an idea of how old a squirrel is at the time of assessment. During the assessment squirrels were assigned to an age group (juvenile, subadult, adult) as described in Table 6 (p. 44). For the purpose of this chapter adult squirrels were generally assumed to be one year old the first time they were captured, to avoid overestimating their age, despite it being possible that some ERS were older than that at first assessment. It will be flagged up when discussing individual ERS, if there are factors implying that the squirrel may have been older than that at first assessment. It is important to note that following a squirrel for two years after it has reached adulthood may already cover 50% to its total expected lifespan in the wild (3-5 years), and at least 20% of its total possible lifespan (10 years). ERS seen before and following the development of clinical signs of leprosy, were used to estimate the time window within which the incubation period for leprosy in ERS might fall.

5.3. Results

5.3.1. Leprosy status of ERS seen more than once

Brownsea

On BI 31 ERS were assessed more than once over the two-year study period. All animals were already adults at the time of initial assessment. Fifteen were male and 16 female. Two ERS were seen in every assessment session, thus a total of five times. Another two ERS were seen four times each, 12 were seen three times and 15 only twice (Figure 68).

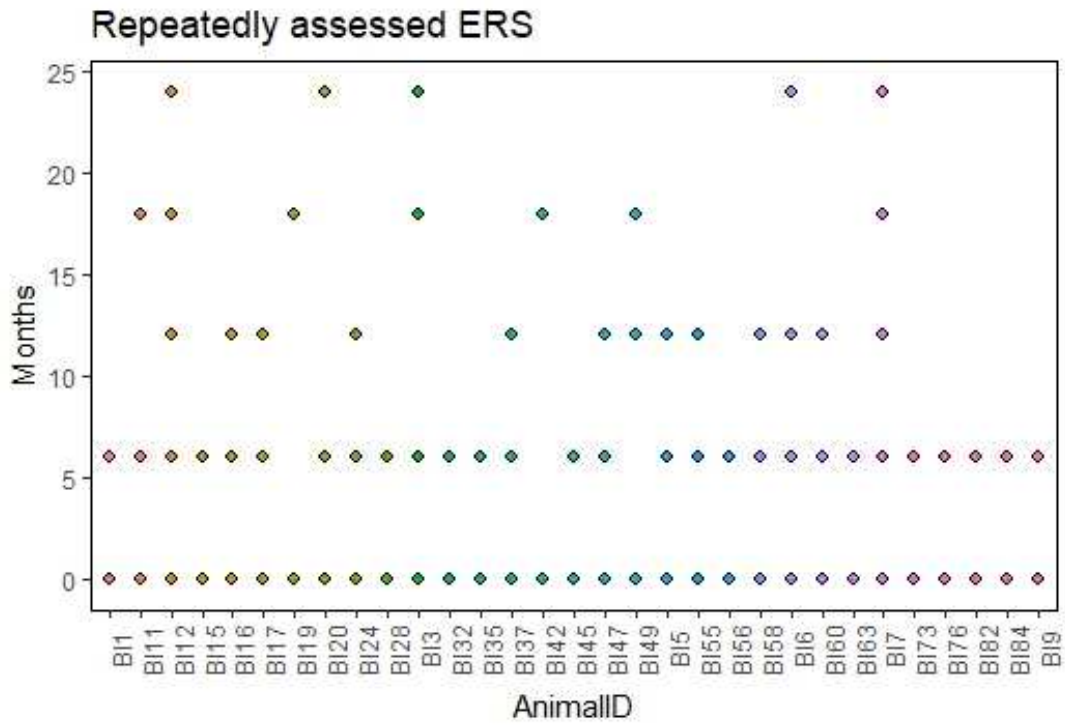


FIGURE 68: NUMBER OF TIMES AND TIME POINTS AT WHICH THE 31 ERS ASSESSED IN MORE THAN ONCE ON BI WERE SEEN.

In nine ERS clinical lesions indicating leprosy were observed at least once. In six ERS it was possible to follow the development of their lesions over time, four of them already showing clinical signs at the initial assessment and two developing them within six months from the initial assessment. In the other three the lesions were only present the last time they were seen (12 and twice 24 months after initial assessment, respectively), thus mainly providing information towards how long an ERS may remain free of clinical signs in this population (Figure 69).

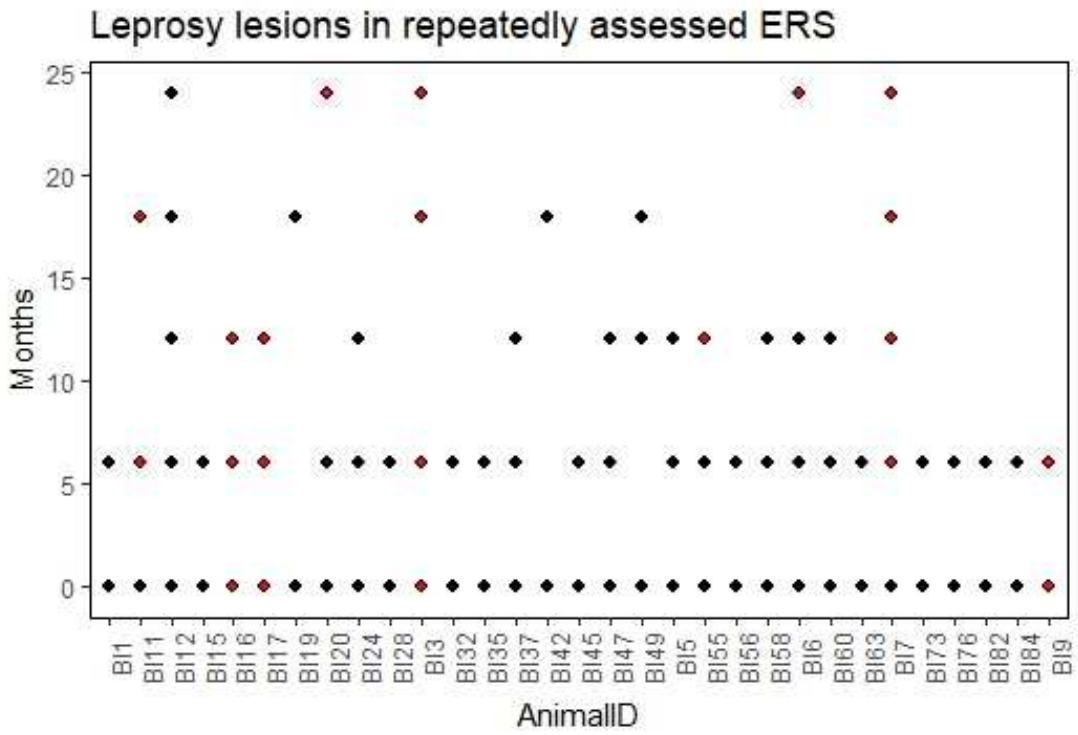


FIGURE 69: PRESENCE (RED) AND ABSENCE (BLACK) OF LEPROSY LESIONS ACROSS THE ASSESSMENTS IN RETURNING ERS

Serum samples were collected and analysed using the αPGL-I UCP-LFA described in chapter 3 (p. 75) for all assessments. Results were noted as positive (serum PGL-I ratio equal or greater than 0.1) or negative (serum PGL-I ratio less than 0.1). For some ERS results correlated well with the presence of clinical lesions, in others they did not (Figure 70). This will be addressed in more detail for each animal below (p. 148).

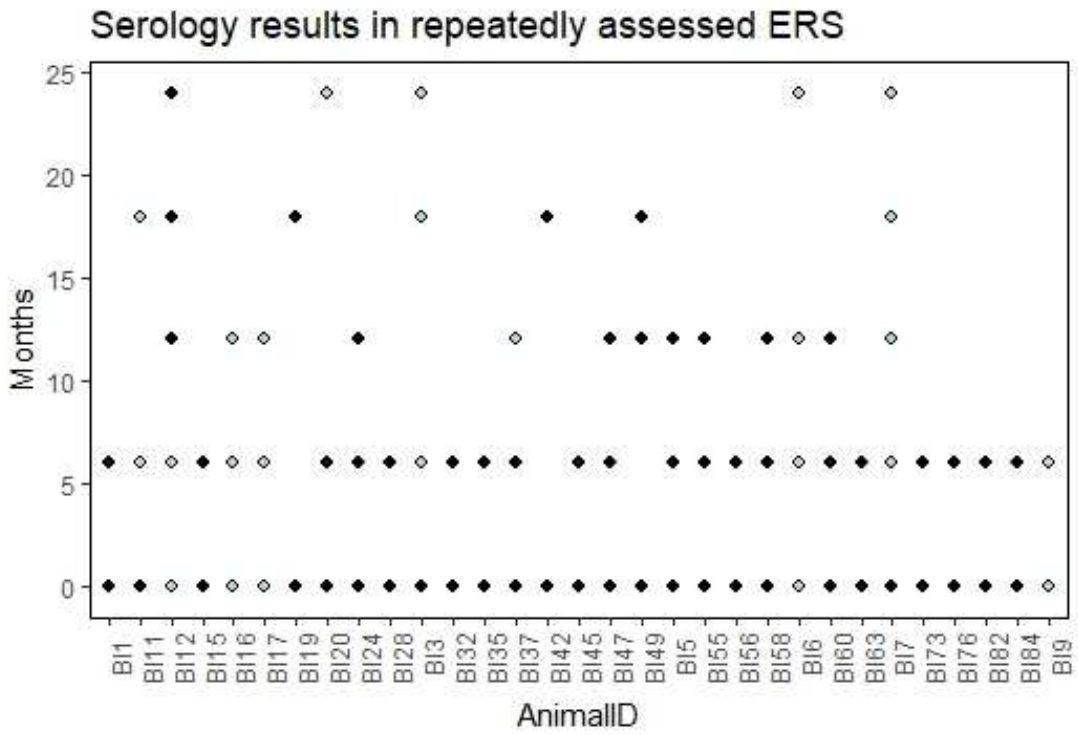


FIGURE 70: POSITIVE (LIGHT BLUE) AND NEGATIVE (BLACK) ALPHA-PGL-I SEROLOGY RESULTS ACROSS THE ASSESSMENTS IN RETURNING ERS

Tissue samples were not collected at all assessments, as previously explained in chapter 3 (p. 72 et seqq.). PCR results were thus unfortunately only available for some assessments in the ERS included in this chapter.

No PCR information was available for five ERS included here. For 14 ERS one PCR result was available and for the remaining 12 ERS two results were available. In eight the result was the same at both assessment times, for four it differed (Figure 71).

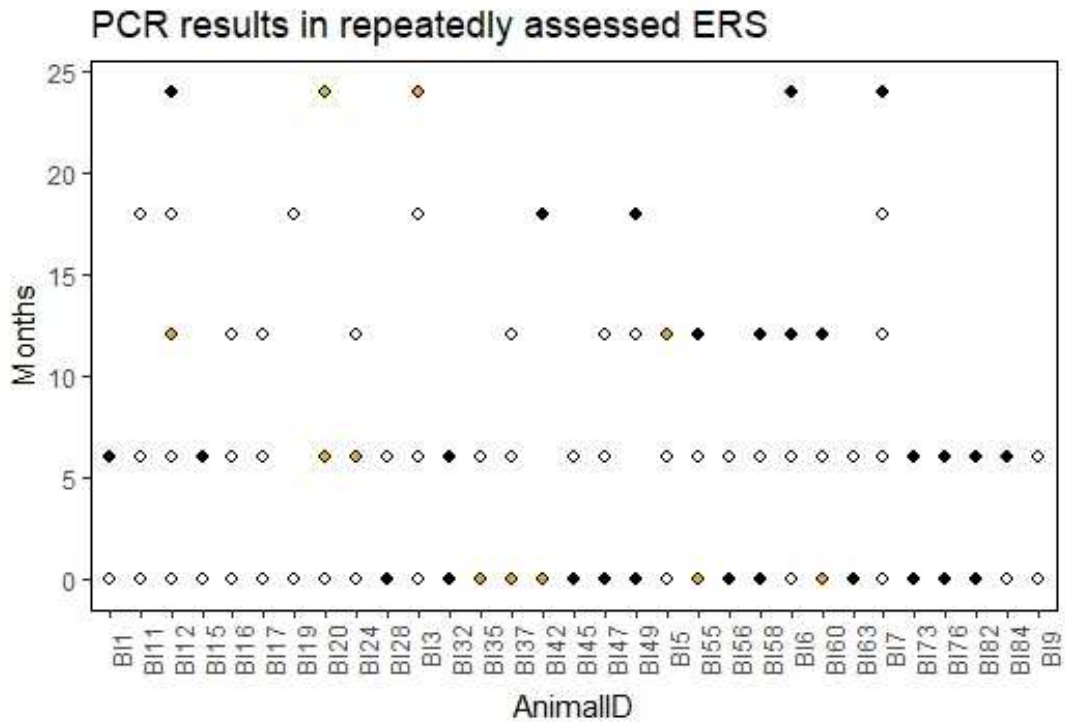


FIGURE 71: *M. LEPRAE* DNA WAS ISOLATED FROM 10 ERS (ORANGE), ONLY IN ONE ERS TWICE. BLACK DOTS = NO DETECTION OF *M. LEPRAE* DNA IN THE PCR, WHITE DOTS = NO TISSUE SAMPLING

Based on the three diagnostic tests (clinical assessment, serology, PCR), eight animals were diagnosed as leprosy cases, at least in their final assessment. Not all of them were identified as colonised before, and not all ERS diagnosed as colonised in the initial assessment developed clinical leprosy within the study period. Most ERS were only classed as contacts, though in some instances no PCR result was available as discussed above and thus not all tests for full diagnosis performed (Figure 72). It is therefore possible, that the proportion of colonised ERS was underestimated.

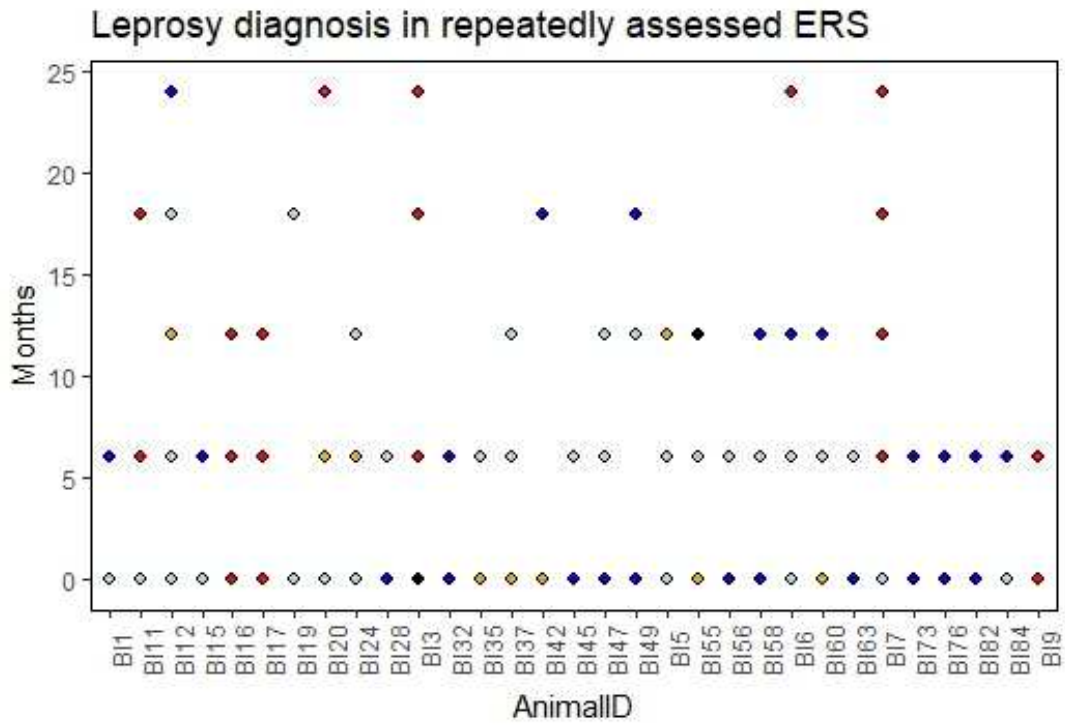


FIGURE 72: LEPROSY DIAGNOSIS IN REPEATEDLY ASSESSED BI ERS. RED= LEPROSY CASE, ORANGE= COLONISED, DARK BLUE= CONTACT, LIGHT BLUE= CONTACT, BUT NO PCR RESULT AVAILABLE AT THIS TIME POINT, BLACK= SUSPICIOUS, ADDITIONAL TESTS NEEDED TO FULLY CLARIFY STATUS.

All ERS assessed repeatedly were and remained in good health, though minor or healed injuries were observed in some, as is to be expected in free ranging wild animals. Only one ERS was classed as acutely unwell, with improvement being likely (Figure 73). In this male a scrotal abscess was present that was cleaned during the assessment.

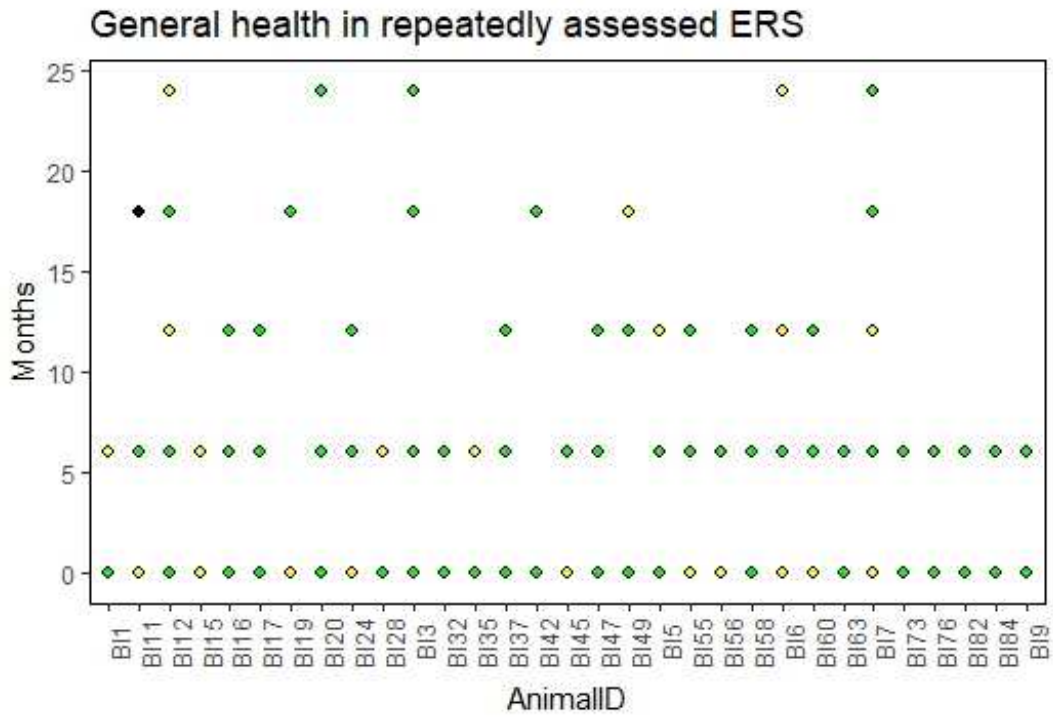


FIGURE 73: GHS IN REPEATEDLY ASSESSED ERS. GREEN = IN GOOD HEALTH; YELLOW: IN GOOD HEALTH, BUT MINOR OR HEALED INJURY PRESENT; BLACK = ACUTELY UNWELL, IMPROVEMENT LIKELY

Reproductive status, body condition and weight will be discussed with respect to the individual animals. These parameters are likely to be influenced by the season in which an ERS was sampled, and underlying individual variation. However, all ERS were in thin or good condition across all assessments included here. It is worth remembering that good internal fat cover was seen post mortem in ERS classed as thin and that this BCS category is therefore no immediate cause for concern, if no other factors imply poor condition in an ERS.

Arran

On AR, nine ERS were trapped and assessed in more than one session. Eight ERS were seen twice (three male, five female), one female was seen three times. All animals were already adult at the time of their first assessment. Four ERS were seen for the second time six months after the initial assessment, three were seen again after 12 months, one after 18 months. The animal re-assessed twice was seen 12 and 18 months following the initial assessment.

No leprosy lesions or seropositivity were seen in any of the animals. *M. leprae* DNA was only isolated from a single animal, and only at the second time this animal was assessed. Thus, while this animal was diagnosed as being colonised by *M. leprae*, all the others were deemed to only potentially have had contact with leprosy bacilli at some point without

becoming colonised or infected. Leprosy is endemic to the AR squirrel population albeit rare and apparently without causing clinical disease (see chapter 6, p. 175).

With only a single change of leprosy status and no further follow up after the change occurred, the AR population did unfortunately not contribute any data to the progression of leprosy over the two-year study period.

5.3.2. Effects of leprosy on individual ERS

Leprosy cases

Eight out of the 31 ERS were identified as leprosy cases. Five of them were male, three female. In another female (BI 55) leprosy was strongly suspected, but the clinical diagnosis could not be confirmed. This individual is still included in the initial overview of cases and presented in detail under the subheading unconfirmed case (p. 155). To provide an overview of disease progression, leprosy lesion category changes over time in these nine ERS were summarised. Figure 74 shows that development of lesions in ERS apparently healthy at initial assessment can occur within six months (BI 7, BI 11), or can take 12 months or longer (BI 20, BI 6, BI 55). Lesion severity did always increase with time. While animals were seen with mild lesions at two consecutive assessments, animals with mild to moderate lesions did usually progress to severe lesions within the next six months. Six squirrels were classed as severely affected but still fit for release. Three squirrels with severe lesions were seen six months after initially being classed as severely affected, and still deemed well enough for release.

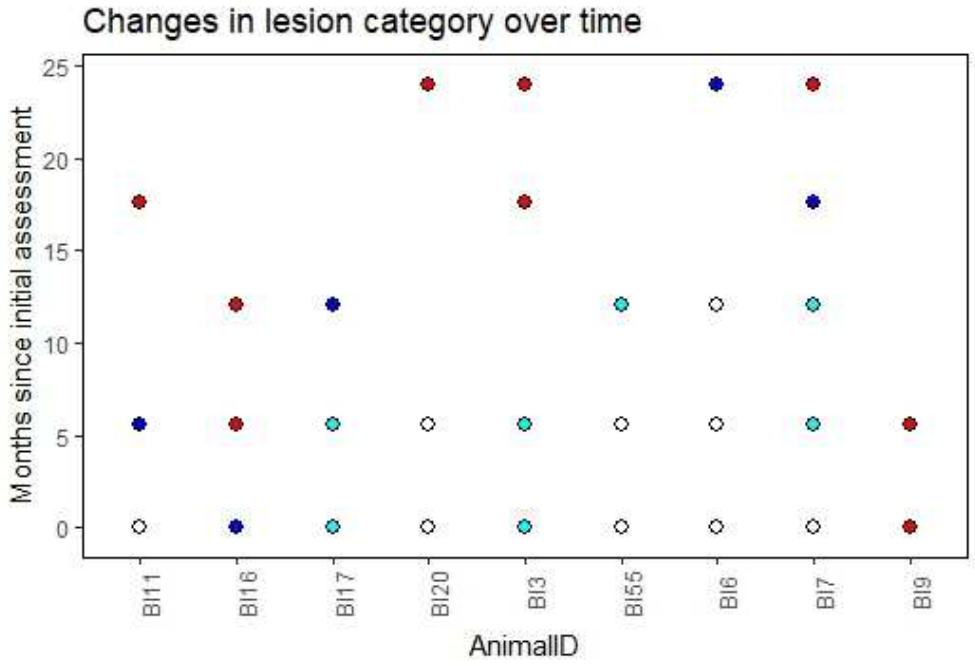


FIGURE 74: CHANGES IN LESION CATEGORY OF LEPROSY CASES, AND THE SUSPECT CASE BI55, SINCE THE INITIAL ASSESSMENT. DOTS REPRESENT THE LESION CATEGORY ASSIGNED TO THE ERS (WHITE= NO LESION, LIGHT BLUE= MILD, DARK BLUE= MILD-MODERATE, RED= SEVERE)

It clearly is possible to observe changes in the leprosy status of squirrels within a two-year time period. The progression of leprosy appeared to have certain patterns, for example that lesions only increased and became more severe with time, but there was individual variation in how quickly clinical disease developed and advanced over time.

Leprosy cases and suspect cases were seen in different reproductive states over time. The presence of clinical signs of leprosy did not prevent reproductive activity nor was it associated with continued weight loss or an irreversible reduction of BCS over time. Eight of the nine ERS in this group had a higher weight in their last assessment, i.e. when they were older, than in the initial assessment. It may be relevant to consider that ERS may fill their stomachs with up to 50g of food (post mortem observation made during this study). Thus, consuming large amounts of bait may have a huge impact on the weight an ERS presents with in a single assessment. Additional variation occurred over time and seasons, being particularly marked in reproductively active females. The data also underlined that BCS and weight are independent variables in ERS, just as in other species (Sakaguchi, 2009). While an individual ERS's BCS varied over time, no general trend to lose body condition over time appeared to be present in these animals with clinical signs of leprosy (Table 24, p. 151).

The lesion score, αPGL-I, and PCR results for each case ERS are graphically illustrated in Figure 75 (p. 152). Where an ERS was seen in consecutive assessment sessions, its lesion score and αPGL-I values are connected by a line. When an ERS missed an assessment time point, a gap is left. Turquoise dots imply at which times tissue sampling and PCR assessment took place and indicate their outcome as either positive or negative.

TABLE 24: REPRODUCTIVE STATUS, BCS AND WEIGHT OF LEPROSY CASES AND SUSPECT CASES OVER TIME

ERS Parameter	Time point 0	6 months	12 months	18 months	24 months
BI 3, adult, male					
Reproductive status	Abdominal testes	Scrotal pigment	Not seen	Scrotal pigment	Scrotal testes
BCS	Normal	Normal		Thin	Normal
Weight	300g	310g		325g	315g
BI 6, adult, male					
Reproductive status	Abdominal testes	Scrotal pigment	Abdominal testes	Not seen	Abdominal testes
BCS	Normal	Normal	Thin		Normal
Weight	300g	290g	300g		300g
BI 7, adult, male					
Reproductive status	Abdominal testes	Scrotal pigment	Abdominal testes	Scrotal pigment	Scrotal testes
BCS	Normal	Normal	Thin	Normal	Thin
Weight	310g	315g	Not assessed	320g	320g
BI 9, adult, female					
Reproductive status	Lactating	Pregnant	Not seen	Not seen	Not seen
BCS	Thin	Normal			
Weight	345g	390g			
BI 11, adult, male					
Reproductive status	Scrotal testes	Scrotal pigment	Not seen	Scrotal pigment	Not seen
BCS	Normal	Normal		Thin	
Weight	320g	335g		350g	
BI 16, adult, female					
Reproductive status	Inactive	Lactating	Inactive	Not seen	Not seen
BCS	Normal	Thin	Thin		
Weight	305g	360g	330g		
BI 17, adult, female					
Reproductive status	Inactive	Lactating	Inactive	Not seen	Not seen
BCS	Normal	Thin	Normal		
Weight	350g	345g	375g		
BI 20, adult, male					
Reproductive status	Scrotal testes	Scrotal testes	Not seen	Not seen	Scrotal testes
BCS	Normal	Normal			Normal
Weight	310g	310g			340g
BI 55, adult, female					
Reproductive status	Inactive	Inactive	Inactive	Study ended	
BCS	Normal	Normal	Normal		
Weight	280g	275g	320g		

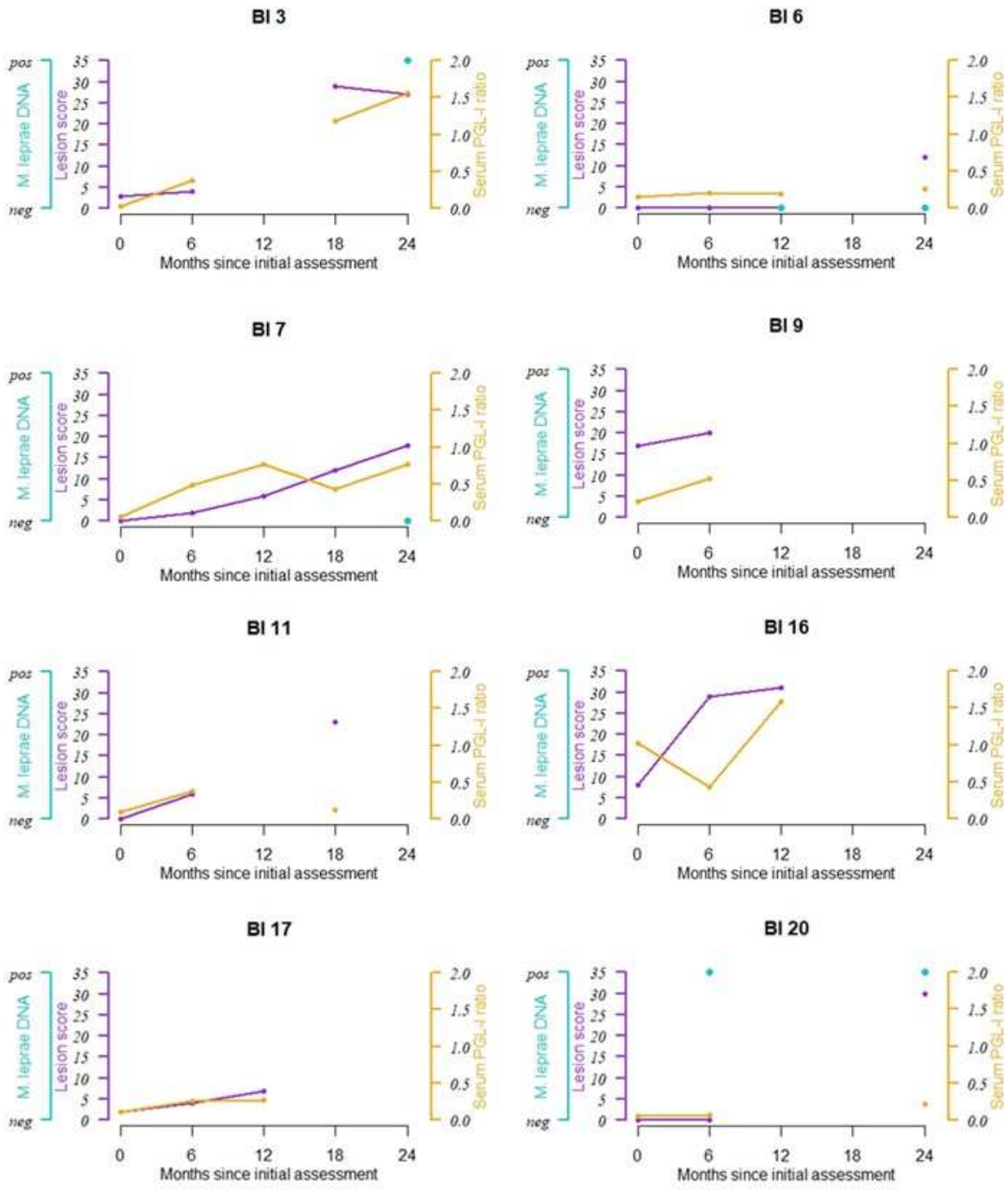


FIGURE 75: LEPROSY LESION SCORE (PURPLE) AND SERUM ALPHA PGL-I RATIO (ORANGE) FOR THE LEPROSY CASE ERS SEEN OVER TIME. TURQUOISE DOTS INDICATE TIME POINTS AT WHICH TISSUE SAMPLES WERE COLLECTED AND WHETHER THEY WERE POSITIVE OR NEGATIVE FOR THE PRESENCE OF *M. LEPRAE* DNA.

In BI 3 a mild leprosy lesion on the right ear (cat mild, score 3) was already present in the initial assessment. However, at this time αPGL-I was still below the threshold for positivity. While the lesion had only mildly progressed six months later (cat mild, score 4), αPGL-I was now above the threshold for positivity. When next seen (18 months post initial assessment)

lesions had intensified, (cat severe, score 29), now affecting both ears and hocks, with an ulceration of the lesion on the left ear. The ulceration had healed in the final assessment (cat severe, score 27), though lesions had increased in size and additional small lesion had formed on the left eyelid. A small abrasion was present, again on the left ear. Its appearance was not quite the same as usually seen in ulcerating lesions and was thus deemed to be traumatic. Therefore, the animal scored overall slightly lower in the final assessment than before, while its αPGL-I ratio was constantly increasing with time. In the final session a tissue sample was collected and the presence of *M. leprae* DNA confirmed.

In BI 6 no leprosy lesions were present in the first three assessments. However, throughout this time the αPGL-I was slightly above the threshold for positivity (0.16, 0.21, 0.19). When the animal was last seen, 24 months after the initial assessment, it had developed mild-moderate leprosy lesions on both ears and the scrotum (score 12), the αPGL-I ratio was slightly higher than before (0.26). Tissue samples were collected twice from this animal, before it developed clinical lesions and when lesions were present. However, no *M. leprae* DNA was isolated at either time point. Tissue samples were not collected from the lesion, as the punch used was not suitable for sampling bulbous lesions, but from a nearby ear section of normal thickness.

At the initial assessment of BI 7 no leprosy lesions were present and the αPGL-I ratio was below the threshold for positivity. Six month later a mild lesion (score 2) had appeared on the left ear and the αPGL-I ratio was clearly above the threshold for positivity (0.48). Twelve months after the initial assessment lesions were still mild and present on the left ear and left hock (score 6) and the αPGL-I ratio had increased (0.77). Eighteen months after the initial assessment lesions had intensified to mild-moderate (score 12) and were obvious on the left ear and hock, and another lesion was thought to be in the process of forming on the right ear. The αPGL-I ratio was lower than before, though still above the threshold for positivity (0.44). After 24 months lesions had become severe, as a small ulceration was present on the lesion of the left ear (score 18) and lesions were now obvious on both hocks. However, the right ear still did not show a fully developed lesion and the change on this ear was not classified as a clear leprosy lesion this time. The αPGL-I ratio had increased again (0.76). It was not possible to isolate *M. leprae* DNA from a tissue sample collected at this time. However, sampling was again restricted to an area adjacent to the lesion due to the punch used.

In BI 9 leprosy lesions present were severe at both assessments (score 17 and 20, respectively) and the αPGL-I ratio was above the threshold for positivity (0.23 and 0.53, respectively). In the first assessment a dry ulcer was present on the left ear, and lesions visible on both ears and hocks. The situation was very similar six months later, the initial ulceration had healed but a new ulcer was present in a different area of the lesion, and the

size of some of the lesions had slightly increased. Additionally, a lesion was now present on the right ear as well.

No leprosy lesions were observed when B11 was first seen and its αPGL-I ratio was just below the threshold for positivity (0.9). Six months later mild to moderate lesions were present, with a typical lesion on the left ear and slight swelling of muzzle and nose. At this point the αPGL-I ratio was clearly above the threshold for positivity (0.39). Eighteen months after the initial assessment the lesions were severe and present on both ears and hocks. At this time a scrotal abscess was observed and the ERS classed as acutely unwell, however, with a good prognosis, particularly after the abscess had been cleaned during the assessment. It cannot be excluded, that a lesion had been present in the area of the scrotum in which the abscess presented. Interestingly, the αPGL-I ratio was barely above the threshold for positivity at this point in time (0.13).

At the time of initial assessment, the leprosy lesions seen in BI 16 were mild to moderate (score 8) only affecting both ears. The αPGL-I ratio was high compared to other animals (1.03). Six months later, lesions had become severe (score 29) with dry ulcerations on both ears and additional lesions on both hocks. The αPGL-I ratio had dropped (0.43) but remained above the threshold for positivity. At this time the animal would have been considered for euthanasia, as we did not yet know that ulcerations could heal. The fact that it had dependent young/was lactating and not returning to them may cause them to starve was the reason it was released. Another six months later it was confirmed that this decision had been correct and it was evident that ulcerations could heal. While the lesions on both hocks and the ears had slightly increased in size, ulcerations were no longer present (score 31). The αPGL-I ratio had risen again (1.59).

In the initial assessment of BI 17 a mild lesion (score 2) was present on its right ear. Initially, the αPGL-I ratio was barely above the threshold for positivity (0.11). Six months later the lesion score had increased to 4 as the lesion had increased in size, and the αPGL-I ratio was increased to 0.26. When seen 12 months after the initial assessment the lesion had further increased in size and an additional small lesion had formed on the left eyelid (mild to moderate lesions, score 7), and the αPGL-I ratio was again slightly higher than before (0.28).

BI 20 appeared to be in good health throughout all assessments, though in the last a toe that might have been previously broken was noted on the left hindfoot. No tissue was collected in the initial assessment, but six month later a sample was taken. At this time BI 20 was already colonised by *M. leprae*, but no clinical signs of disease were present and the αPGL-I ratio was well below the threshold for positivity (0.06 and 0.07, respectively). Eighteen months later leprosy lesions were present on both ears, the left hock and the scrotum (severe, score 30). On the right ear and scrotum dried ulcers were present on the lesions.

The αPGL-I ratio was above the threshold for positivity (0.23), and *M. leprae* DNA could again be isolated from a tissue sample collected from the non-swollen area of one ear.

Unconfirmed case

During the initial assessment of BI 55 no leprosy lesions were noted and the αPGL-I ratio was 0. However, *M. leprae* DNA was isolated from a tissue sample collected and the animal diagnosed as colonised by *M. leprae*. Six months later the clinical and serological situation was unchanged, but no tissue sample was collected. The αPGL-I ratio remained at 0, and from a new tissue sample collected 12 months after initial assessment from the opposite ear no *M. leprae* DNA was isolated (Figure 76). At this time a mild single clinical lesion (score 5) was seen on the right hock (Figure 77).

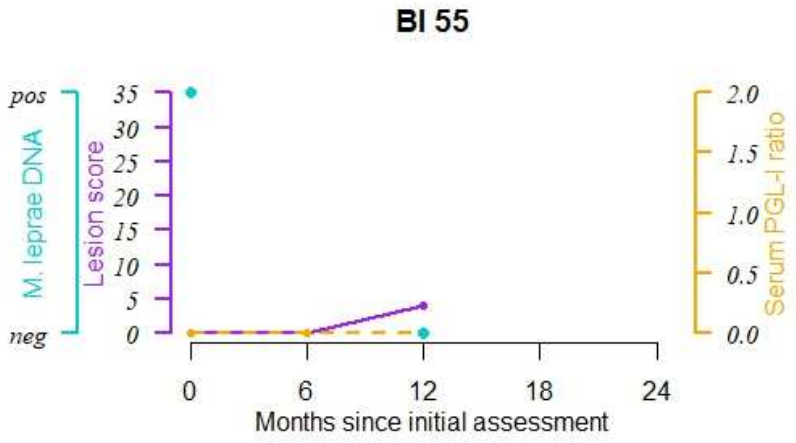


FIGURE 76: LEPROSY LESION SCORE (PURPLE) AND SERUM ALPHA PGL-I RATIO (ORANGE) FOR BI 55 OVER TIME. TURQUOISE DOTS INDICATE TIME POINTS AT WHICH TISSUE SAMPLES WERE COLLECTED AND WHETHER THEY WERE POSITIVE OR NEGATIVE FOR THE PRESENCE OF *M. LEPRAE* DNA.

FIGURE 77: LESION OBSERVED ON THE RIGHT HOCK OF BI 55. CONSISTENCY, HAIR LOSS, AND BULBOUS NATURE ARE IN LINE WITH THE PRESENTATION TYPICAL FOR A LEPROSY LESION.

As the lesion was typical and the squirrel resided in an area in which clinical leprosy cases were regularly occurring, it was still suspected that this was a leprosy case, however, without the laboratory confirmation it could only be called suspicious. Re-testing/additional assessment at a later point in time, had it been possible, would have been likely helpful in clarifying whether this was a case of leprosy or not.

In summary, the following important observations were made in the leprosy cases and suspect cases:

- Lesion progression is highly individual. It can be slow (BI 7, BI 9, BI 17), but in some instances more rapid intensification of lesions occurs (BI 3, BI 16)
- Ulcerations on leprosy lesions can heal without treatment (BI3, BI 9, BI16)
- *M. leprae DNA* can be detected up to 12 or even 24 months before clinical signs of leprosy are documented (BI 20, BI 55)
- It is possible, that *M. leprae* DNA is detectable before the onset of clinical signs, but is not detectable when clinical signs are present (BI 7, BI 6, BI 55)
- The αPGL-I ratio increases as clinical signs of leprosy intensify in most ERS however, values may fluctuate throughout the progression of the disease (BI 7, BI 11, BI 16)
- In some ERS the αPGL-I ratio may be above the threshold of positivity before clinical signs of disease are obvious. However, the ratio increases further when lesions develop (BI 6)

Colonised ERS

Seven squirrels were classified as colonised by *M. leprae* in at least one of their assessments, based on the isolation of *M. leprae* from an ear tissue sample. Four were male, three female. Leprosy colonised ERS were also seen in different reproductive states over time. The colonisation with leprosy bacilli was not associated with continued weight loss or an irreversible reduction of BCS over time. Six of the seven ERS in this group had a higher weight in their last assessment, than in the initial assessment. Again, the highest variations in body weight were seen in reproductively active females. No general tendency to lose body condition over time appears to be present in colonised ERS (Table 25, p. 157).

The same graphic format as in leprosy cases was chosen to display lesion score (always 0), αPGL-I, and PCR results in colonised ERS, to allow for a more intuitive comparison of the two groups. Again, lesion score and αPGL-I values are connected by a line, when an ERS was seen in consecutive assessment sessions, and a gap was left when a session was missed. Turquoise dots imply at which times tissue sampling and PCR assessment took place and their outcomes as either positive or negative (Figure 78, p. 158).

TABLE 25: REPRODUCTIVE STATUS, BCS AND WEIGHT OF ERS COLONISED BY *M. LEPRAE*

ERS / Parameter	Time point 0	6 months	12 months	18 months	24 months
BI 5, adult, male					
Reproductive status	Abdominal testes	Scrotal pigment	Scrotal testes	Not seen	Not seen
BCS	Normal	Normal	Normal		
Weight	325g	340g	335g		
BI 12, adult, female					
Reproductive status	Lactating	Pregnant	Lactating	Inactive	Inactive
BCS	Normal	Normal	Thin	Normal	Normal
Weight	355g	325g	345g	365g	325g
BI 24, adult, female					
Reproductive status	Inactive	Lactating	Inactive	Not seen	Not seen
BCS	Normal	Normal	Normal		
Weight	320g	390g	360g		
BI 35, adult, male					
Reproductive status	Scrotal testes	Abdominal testes	Not seen	Not seen	Study ended
BCS	Normal	Thin			
Weight	270g	295g			
BI 37, adult, female					
Reproductive status	Inactive	Pregnant	Inactive	Not seen	Study ended
BCS	Normal	Thin	Thin		
Weight	300g	385g	335g		
BI 42, adult, male					
Reproductive status	Scrotal testes	Not seen	Not seen	Scrotal testes	Study ended
BCS	Normal			Thin	
Weight	270g			290g	
BI 60, adult, male					
Reproductive status	Scrotal testes	Scrotal pigment	Abdominal testes	Study ended	
BCS	Thin	Thin	Thin		
Weight	290g	310g	295g		

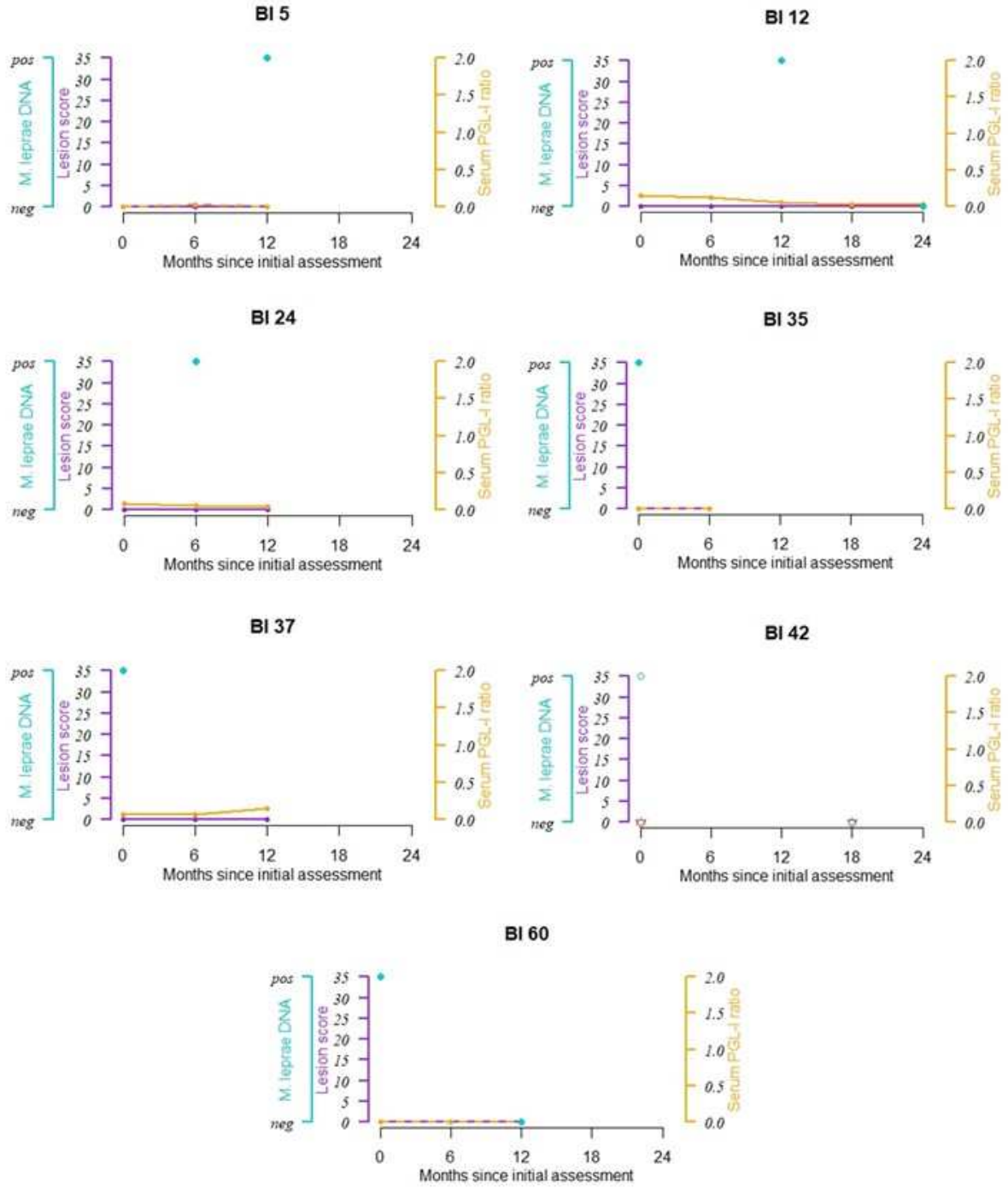


FIGURE 78: LEPROSY LESION SCORE (PURPLE) AND SERUM PGL-I RATIO (ORANGE) FOR THE LEPROSY COLONISED ERS SEEN OVER TIME. TURQUOISE DOTS INDICATE TIME POINTS AT WHICH TISSUE SAMPLES WERE COLLECTED AND WHETHER THEY WERE POSITIVE OR NEGATIVE FOR THE PRESENCE OF *M. LEPRAE* DNA. IN BI 42 LESION SCORE AND ALPHA-PGL-I RATIO WERE 0 AT BOTH ASSESSMENTS AND THE PCR NEGATIVE AT 18 MONTHS.

In BI 5's initial assessment a small bulbous lesion under the chin was noted and initially suspected to be an unusual leprosy lesion. However, the αPGL-I ratio at this time was zero. The lesion had disappeared six months later, the αPGL-I ratio was still close to 0 (0.03). As a disappearance of leprosy lesions is not yet described for ERS and this lesion was unusual to

begin with, it was assumed that it had been caused by something other than leprosy bacilli. In the third assessment after 12 months no clinical lesions were present and the αPGL-I ratio was 0. However, at this time a tissue sample was collected and *M. leprae* DNA isolated. Unfortunately, the ERS did not return again in the later sessions of this study.

At the first two assessments of BI 12, the αPGL-I ratio was slightly above the threshold for positivity (0.15; 0.12), in the later assessments it was below. No clinical lesions indicating leprosy were seen in this squirrel during the two years of the study. Half-way through *M. leprae* DNA was isolated from a tissue sample, however, not from a second sample taken at the end of the study.

At no point were skin lesions noted in BI 24. The serum αPGL-I ratio remained close to 0 and below the threshold for positivity. The animal was identified as being colonised by *M. leprae* six months after the initial assessment, but did not develop lesions within the next six months. Unfortunately, it did not return in the fourth and fifth sampling session. It remains thus unknown if it developed lesions at a later time.

From a tissue sample collected during the initial assessment of BI 35 *M. leprae* DNA was isolated, and the squirrel classed as colonised. At both sampling times the αPGL-I ratio was 0 and no clinical lesions indicating leprosy were present. This was a second example of an ERS colonised by *M. leprae* and remaining healthy for at least six months afterwards.

At no time did BI 37 show any clinical signs of leprosy. However, following the initial assessment *M. leprae* DNA was isolated from a collected tissue sample, implying that it was at this time colonised by the bacteria. At the initial and six months assessment the αPGL-I ratio was below the threshold for positivity, but above this threshold 12 months after the initial assessment (0.15). Unfortunately, no tissue was collected at this time and the squirrel could not be assessed again to see if it would develop lesions. It is however interesting to note, that the colonisation with *M. leprae* in this animal did not result in clinical disease over twelve months.

In neither assessment of BI 42 were any skin lesions indicating leprosy observed. The αPGL-I ratio in its serum was 0 both times. However, in the first assessment, *M. leprae* DNA was isolated from the collected tissue sample.

BI 60 is similar to BI 42, though for this animal three assessments are available covering 12 months. No lesions indicating leprosy were seen at any time and the αPGL-I ratio was consistently 0. However, from a tissue sample collected during the first assessment *M. leprae* DNA was isolated. In the third assessment another tissue sample was taken, from which no *M. leprae* DNA could be isolated.

In summary, the following observations were made in *M. leprae* colonised ERS:

- Colonised ERS may stay free from clinical signs of leprosy for six to 18 months after being identified as colonised (BI 12, BI 24, BI 35, BI 37, BI 42, BI 60)
- Colonised ERS may test negative in a later PCR assessment (BI 12, BI 42, BI 60)
- Colonised ERS may occasionally have αPGL-I ratios above the threshold for positivity (BI 12, BI 37)
- Colonised ERS with αPGL-I ratios transiently above the threshold for positivity may still not develop clinical leprosy within a year (BI 12)
- The αPGL-I ratio is very helpful in clarifying whether an unusual skin lesion is likely to actually be caused by leprosy bacilli (BI 5)

Leprosy contacts

Fifteen squirrels were seen more than once (12 twice, three thrice), without showing any clinical signs of leprosy and the αPGL-I ratio consistently being 0 or below the threshold for positivity. For 14 of these animals' tissue samples were collected at least once, for six even twice. From none of these tissue samples could *M. leprae* DNA be isolated. In 11 animals the two assessments were only 6 months apart, but for two each the assessments covered 12 months and 18 months.

Six of these squirrels were male, nine female. All of them were in good health and in normal or thin body condition. As they were trapped in the same area of BI as the diseased and colonised squirrels, it is likely that all of these ERS may have at some point in their life had contact to leprosy bacilli and/or infected ERS. It cannot be excluded that they were colonised or infected with very low bacterial loads at this point, but based on the currently available methods these ERS were defined as leprosy contacts, and in those instances where both PCR and serological results were available, it could be cautiously said that they appeared unlikely to be affected by leprosy.

5.4. Discussion

The development and progression of clinical leprosy lesions was successfully documented in one (BI) of two ERS populations within a two-year timeframe. In nine (29%) out of 31 ERS assessed several times, disease development and/or progression was observed. Data covering the full two-year time frame was only available for four of them, showing that even in shorter timeframes changes can be observed.

Lesion progression could be followed in six ERS for six to 24 months (mean 14 months, SD= 5.7) in this study. All of these ERS were deemed to be fit for release and likely to continue to survive and thrive each time they were seen. Disease progression in other hosts is slow, illustrated by the fact that several years may pass following the onset of clinical disease in humans before clinical signs are obvious enough to make the clinical diagnosis (Li *et al.*, 2016; Ferreira *et al.*, 2018) and by an average survival time of more than 2.5 years following

experimental infection in NBA (Storrs *et al.*, 1974). Based on the wide variety of clinical leprosy presentations and progression documented in other host species (Truman, 2005; Lastória and de Abreu, 2014b; Smith *et al.*, 2015), it was assumed that there would be individual variation in the progression of leprosy in ERS as well. This assumption is supported by the data presented here. It is interesting to observe that while three ERS were classed as mild twice at six months intervals, and as many classed twice as severe, a mild-moderate state was not noted more than once in any ERS included in this dataset and only seen in four ERS at all. No ERS with moderate lesions was seen within this longitudinal dataset. This could imply that lesion development starts quite slowly, the ERS remaining mildly affected for six month or longer, then transitions through mild-moderate and moderate stages slightly faster. After lesions become severe ERS seem able to thrive over another extended period of time. True regression of lesions was not observed in any of the ERS in this study. An apparent regression may occur when ulcerations heal. This implies that the total body score established in chapter 3 is not yet perfectly able to document the progression of lesions, as the sudden score increase that was integrated for ulcerations due to their potential welfare implications, may be too high. Together, this could reflect a need to further improve the leprosy lesion categorisation system, for example by adjusting the score assigned for ulcerated lesions, joining the mild-moderate and moderate categories as one transitional stage and adding another category above severe lesions. This should describe a state in which obvious additional health impairments with a negative welfare impact, such as severe secondary conditions and emaciation are present. However, such an adaption would ideally be based on additional data from more ERS to ensure that alterations are going to lead to a true improvement. Altogether, it is difficult to infer on disease progression in ERS in general from just nine ERS, but it appears that leprosy in ERS is a slow acting, chronic disease, progressing on the scale of months and years comparable to what is described in other hosts.

Interestingly, severe ulcerations were seen on ear lesions of lactating females, with a smaller ulceration being observed during pregnancy. Changes in immune status of females during pregnancy and lactation have been associated with changes of leprosy status in humans (Lockwood and Sinha, 1999; Singh and Perfect, 2007). While ulcerations are not exclusive to female squirrels and not always associated with lactation, postpartum immune reconstitution could be a risk factor for their development. Other risk factors could include acute infections with other pathogens or stressful events like prolonged competition with other ERS for scarce resources, for example food or mating partners. Targeted research and long-term monitoring of individuals would be necessary to further assess these risk factors. In humans ulcers are mainly described as secondary complications on the extremities following neurological damage and loss of sensitivity, that may continue to occur even after the original leprosy infection is successfully treated (Kunst, 2000; Barreto and Salgado, 2010).

Ulcerations to the extremities are also described for TT human patients where RR occur (Soares *et al.*, 2017). Furthermore ulcerations are described to occur on the extremities of NBA and extremities and face (not explicitly ears) of primates (Kirchheimer, 1975; Gormus *et al.*, 1988; Sharma *et al.*, 2013). This implies that differences in the pathogenesis of ulcerations may occur between the host species and these could be addressed in future studies focussing on comparing the host-pathogen interactions in the different species.

Looking at changes in αPGL-I ratios, which are linked to the ERS's immune response to *M. leprae* infection, three ERS (BI3, BI9, BI17) showed the pattern expected following the results presented in chapter 3 (p. 88). Their αPGL-I ratios crossed the threshold for positivity when clinical signs of leprosy developed and the ratio then consistently increased as clinical signs intensified.

In BI6 αPGL-I ratios above the cut off for positivity were seen up to 18 months before clinical lesions developed, and they only slightly increased once clinical signs became apparent. No *M. leprae* DNA could be isolated from tissue samples from this ERS either before or after it developed clinical signs of disease. This could be an individual reactionary pattern to the pathogen presence which resulted in an early humoral immune response and outside the main lesion consistently low bacterial loads in this individual. Future studies should assess whether such patterns occur in more ERS and whether there are any particular individual factors linked to it. The data collected in this study does not allow to speculate further.

An interesting pattern was seen in αPGL-I ratios of three other ERS (BI7, BI11, BI16), that could also explain an observation made in BI20. In these three ERS αPGL-I ratios initially behaved as expected and crossed the threshold for positivity when clinical signs of leprosy were first observed and then increased as the lesions slowly intensified. Then, however, as lesions progressed αPGL-I ratios dropped, staying above the threshold for positivity though, to six month later being higher than before the drop. In the case of BI 7 the drop coincided with the development of lesions in a new body area, while it occurred at the same time as an additional infection/acute abscess was seen in BI11. In BI16 the drop occurred during lactation and while the leprosy lesions were ulcerated. In BI20 detected αPGL-I ratios were unexpectedly low for the severe, ulcerated lesions observed in the third assessment. This could be explained with a generally weak humoral response in this ERS or with a similar temporary decrease in anti-leprosy antibody levels when secondary processes were present. In human leprosy patients αPGL-I levels are reported to persist, unless the bacteria are eliminated (Lastória and de Abreu, 2014b), however, it is not specified whether this excludes fluctuations as long as levels stay above the threshold for positivity as was the case in these ERS. The innate immune response initially determines the progression of leprosy in other hosts (Pinheiro *et al.*, 2018) and potentially influences the following humoral immune response (Fonseca *et al.*, 2017). The humoral immune response can be generally be regulated via many pathways (Taher *et al.*, 2017), and no ERS specific data on its regulation

is currently published. However, there is no indication yet that this is fundamentally different in this species compared to the other hosts. Understanding what causes the αPGL-I ratio fluctuations in ERS could help to identify factors determining the outcome of clinical leprosy in this species. They could also be indicators for other immunomodulatory processes that could have a greater impact on ERS welfare than leprosy itself, for example secondary infections. If this were the case, observing fluctuations could spark further testing on samples from this ERS to assess if other pathogens are present that may be of relevance to the population as a whole.

BI55, the unconfirmed case, makes the argument for repeated testing if leprosy is suspected based on clinical assessment but low or absent αPGL-I are found and PCR results are negative. The clinical lesions were still very mild when linked to a negative αPGL-I result and it has been shown that the humoral response can lag behind the development of clinical lesions (chapter 3). Even more interesting is the observation that the ERS was PCR positive for *M. leprae* DNA before it developed clinical signs of disease but PCR negative when an early lesion was present. It has been seen in other ERS with early leprosy lesions that the PCR is negative when the tissue sample is collected outside the actual lesion (chapter 4) and it is known for humans that PCR results are by far the most reliable when biopsies are collected from an active lesion (Fontes *et al.*, 2018). Future research could address whether there is a particular process underlying the phenomenon where leprosy bacilli are sometimes readily detected in ear tissue before clinical signs develop, but appear limited to the lesions at least at the early stages of clinical disease. Some histological observations of very few bacteria in the tissue right next to a lesion illustrate this phenomenon (Figure 79). Comparative PCR from lesion and adjacent tissue in carcasses displaying early stages of leprosy would allow further insights into the matter.

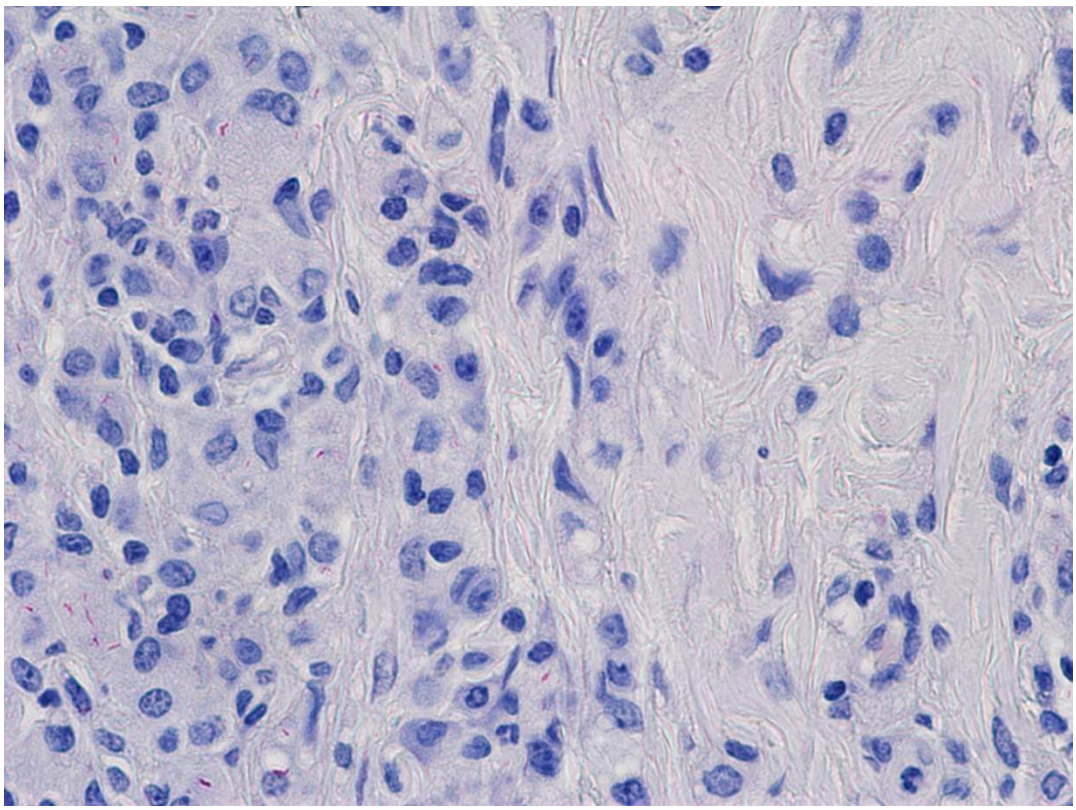


FIGURE 79: AFB IN LEFT HALF OF IMAGE ARE LOCATED WITHIN A CLINICALLY OBSERVABLE LEPROSY LESION, WHILE THEY BECOME VERY SCARCE AND STAIN ONLY FAINTLY IN THE NONINFLAMED ADJACENT TISSUE IN THE TOP RIGHT CORNER OF THE IMAGE (EAR SECTION BI062_17)

Five colonised ERS had αPGL-I ratios levels below the threshold for positivity at all assessment points. For four only one PCR result (positive) was available. One ERS (BI 5) could unfortunately not be followed further as it did not return after the session in which the tissue sample had been collected. Another two (BI 24, BI 35) did not develop clinical signs of leprosy within six months of testing positive for the presence of *M. leprae* DNA. In two (BI 42, BI 60), tissue sampling was repeated after 12 and 18 months respectively, and now no *M. leprae* DNA could be detected. Without following the ERS further it cannot be said whether they will or will not develop leprosy, but these two could be an indicator that clearing of colonisation/subclinical infection, which has been described for other hosts, is possible in ERS as well. Alternatively, it could be speculated that these ERS were just about to develop clinical lesions and the bacteria therefore had become localised and where thus not present in the tissue sampled, or that a different sampling location would have returned a different result, i.e. the negative results being false negatives.

The other two colonised ERS (BI 12, BI 37) had a αPGL-I ratio slightly above the threshold for positivity at one point, but not at the same time point at which they were identified as PCR positive but either before or after. BI12 had a slightly elevated αPGL-I ratio when first seen, a positive PCR 12 month later, and a negative one another 12 months later, at both times having αPGL-I ratios below the threshold for positivity. This could therefore be another

example of a cleared colonisation, but it cannot be determined without following the ERS further. BI37 did not develop clinical signs of leprosy within 12 months of testing positive for *M. leprae* DNA, but had a αPGL-I ratio very slightly above the threshold for positivity in the last assessment. Without following this ERS further, this result cannot appropriately be interpreted.

An onset of clinical signs of leprosy was documented in five ERS in this study, when BI 55 is included. In two clinical signs were obvious six months after their initial assessment, in the other three signs are likely to have taken more than 6, or in one (BI 6) even more than 12 months to develop. Additionally, it was possible to show that six ERS colonised with *M. leprae* did not develop clinical signs of leprosy within the next six to 18 months. It cannot be said whether these ERS will ever develop clinical signs of leprosy, or if they may even be able to eliminate the bacteria, as negative PCR results in a second tissue sample could suggest for BI 12, BI 42 and BI 60. The latter is described for the other host species (Sharma *et al.*, 2018; van Hooij *et al.*, 2018). Still, assuming that the ERS seen in this study became infected as adults, it appears likely, that the incubation period for leprosy in ERS can last between just under six months up to 18 months and longer. If the ERS were already infected while still juveniles (for example through close contact with a mother with ulcerated lesions) the incubation period could be extended by another 9-11 months, i.e. the time it took these ERS to reach adulthood. The incubation period in ERS thus appears similar to that reported for other host species. Descriptions of early lesions of LL human patients will in most instances not capture the very earliest presentations possible, but will document the state in which the patient seeks medical attention. It can thus not be said, whether in such cases single lesions, as seen here in ERS, do not occur and are specific to this newly discovered host, or if that stage is usually just not well documented in people, where LL cases are always described to present with multiple lesions (Virmond, Grzybowski and Virmond, 2015; Gaschignard *et al.*, 2016).

For none of the adult ERS seen in this study an exact age or the time at which it became infected with leprosy bacilli can be known. It is unlikely to be possible to fully correct for these shortcomings in wildlife field studies. However, small, closed populations like BI could offer an opportunity to follow ERS from first emergence from the nest and over their full lifespan, using marking techniques that allow hands-off monitoring of the individuals. Such studies could, in the future, produce data allowing an improved incubation period estimate for ERS. However, given the wide range of time reported for the leprosy incubation period in other hosts, higher precision may just not be possible in such a variably and slow acting disease.

In none of the ERS did leprosy seem to have a clear negative impact on GHS, BCS, weight or reproductive activity. This minimal effect of leprosy infection on an animal host under natural conditions has been observed in NBA as well (Truman, 2005; Loughry *et al.*, 2009).

Three out of the four females had noticeably produced offspring despite the infection, the fourth being slightly lighter and potentially younger than the others. Even severe lesions did not appear to hinder successful mating and pregnancy. However, no information is available on kit survival, which is often low in this species (Bosch and Lurz, 2012). Whether supplementary feeding, which occurs in some areas of BI, mitigated a loss of weight or BCS cannot be assessed based on the current dataset, as no information is available on the extent to which affected ERS made use of such supplementary food sources.

There appear to be marked epidemiological differences in leprosy dynamics in the two populations, these will be addressed in chapter 6. A factor contributing to the differences in progress detection seen in this part of the study is likely to be the proportion of repeatedly trapped ERS. It was much lower on AR, where no changes of clinical status were seen, than on BI (17.3% vs. 42.5%). It was, however, still possible to document an ERS becoming colonised by *M. leprae* in this population, implying that with an extension of the study, further observations might have been made. Another factor could be the climate both populations live in. ERS are different from humans and NBA in an important factor that could have an impact on how an infection with *M. leprae* progresses in this species: they have a higher core body temperature. The low body temperature of NBA had been identified as a core feature making them a suitable host for *M. leprae* and was used to explain why systemic disease occurs in this species (Purtilo *et al.*, 1974; Truman, 2005). It has been suggested that colder temperatures in peripheral body areas allow leprosy bacilli to grow in humans (Virmond, Grzybowski and Virmond, 2015), however, one of the few studies looking at the presence of leprosy bacilli in a variety of human skin areas did not confirm such a pattern (Kaur and Kumar, 1978). Peripheral skin temperature in humans is altered by the ambient temperature and was shown to vary between 29 and 33°C in healthy, young women (Martinez-Nicolas *et al.*, 2015). New methods for the continuous measurement of human skin temperatures have been developed in the last decade (Smith *et al.*, 2010; Webb *et al.*, 2013), but no research applying them in leprosy patients to investigate how temperature changes may influence leprosy progression and bacterial growth has been published. Such data would be needed to determine, whether differences in ambient temperature, leading to changes in skin temperature, could potentially influence leprosy progression in a host. Such studies are easier to conduct in the larger host species, in which sensors of the currently available size can be used. If ERS sized sensors became available, comparing ear temperatures in ERS from BI and AR throughout the year could allow to determine whether a significant difference exists that could explain why more individuals in the population living in a slightly warmer climate, developed clinical disease.

If this study was to be repeated, one major alteration that should be made is including a tissue sample at the initial assessment in all ERS, to be aware whether ERS are already colonised with leprosy bacilli at this point. However, to be able to repeatedly test ERS

without clinical signs for the presence of leprosy bacilli, ear tissue samples may not be suitable, as they can only be taken a limited number of times. One way around this could be to use repeated blood samples to isolate *M. leprae* DNA, although it would be necessary to first assess whether leprosy bacilli DNA can be isolated from 1ml ERS blood samples, as is the case in 3-5ml samples from humans (Wen *et al.*, 2013). Other alterations could aim to increase re-trapping success or to collect data on disease progression without having to handle the ERS. Marked ERS could be followed over time using camera traps combined with microchip readers installed in the area covered by the camera. Re-trapping success could be increased by saving time using trapping protocols with a shorter handling of each ERS (i.e. exclusively clinical assessment of already confirmed leprosy cases in handling cones without anaesthesia) and trapping sessions that are continued until no new previously marked ERS are trapped. Similar protocols are, for example, established for squirrel population monitoring studies (Barkalow, Hamilton and Soots, 1970; Hansen, Nixon and Havera, 1986; Di Pierro *et al.*, 2010).

To overcome the limitations of studying leprosy progression through live sampling, it could also be combined with disease progression modelling. Valuable lessons for model development could be learned from progression models developed for other progressive diseases that can start with mild symptoms, delayed diagnosis, and where disease progression can be highly variable between individuals, for example Alzheimer's (Cook and Bies, 2016). Such models can incorporate both biomarkers (i.e. serological or molecular test results) as well as clinical outcomes summarised in categories (Cook and Bies, 2016). Models exist that can handle variable patient histories and missing entries and are still able to make projections for disease progression beyond the point of initial data collection. Unfortunately, such models require large dataset (several thousand patients) to be trained and evaluated properly (Zhu and Sabuncu, 2018). So, while they would be a very valuable addition in the long run, in the short term not enough information is available to adapt them well for use in squirrel leprosy research. If more longitudinal data can be collected, it might be useful to discuss the dataset to be collected with modellers to see if alterations in the information collection protocol could lead to a quicker development of such tools, that may be useful for managers to assess for how much longer a diseased ERS is likely to survive. If survival is likely to be very limited anyway, humane removal of the individual from the population could be a sensible option and may reduce the number of leprosy bacilli that are being excreted into the environment. In cases where long term survival of a clinically diseased ERS in a small population of high genetic value is likely, the additional generations produced by leaving the ERS in the population outweigh any theoretical reduction in bacterial load or transmission risk in the population. These considerations only stand so long as transmission of leprosy in ERS is not understood and can thus not generally be managed.

Conclusion

While the onset and progression of clinical leprosy can be observed in ERS within a two-year timeframe, an extended incubation period of several months or years along with mostly slow progression of clinical disease appears to be present in this host, just like in NBA or humans. Progression is variable between individuals and may be influenced by additional risk factors that alter the host immune response, such as pregnancy, lactation or secondary infections. ERS can thrive from several months up to years after becoming colonised with leprosy bacilli without developing clinical signs of disease. It could be possible for some ERS to clear a colonisation with *M. leprae*, however, due to the limitations of current PCR assays, very low bacterial loads may just not have been detected in later assessments. This study has offered valuable initial insights into the progression of leprosy. Longer-term studies including larger numbers of ERS, using additional tools to investigate the ERS immune reaction and to more sensitively detect *M. leprae* DNA, potentially also employing modelling methods to make the most of the available data, could still uncover much additional information about host-pathogen interactions in ERS leprosy progression.

Chapter 6: Squirrel leprosy epidemiology

6.1. Introduction

Next to clinical signs and pathology, the epidemiological characteristics of a disease are an integral part of its basic description. To complete the basic description of leprosy in ERS begun in the earlier segments of this study addressing clinical and pathological aspects of leprosy in live ERS, this segment will now focus on disease frequency and distribution.

Only in the British Isles have ERS been identified as infected with leprosy bacilli (Avanzi *et al.*, 2016). Based on opportunistic samples leprosy prevalence (colonisation and clinical infection) in submitted carcasses varied in different locations from very low (~1% on the Isle of Wight, ~5% in Ireland), over moderate (~13% in Scotland) to very high (~100% on BI) (Avanzi *et al.*, 2016; Butler *et al.*, 2017).

Data from the two focus populations (AR, BI) of this study was used to establish the apparent prevalence and morbidity, and to calculate a cautious estimate for the incidence of leprosy in live ERS. It was assessed whether the leprosy prevalence and morbidity are similar in the two populations.

The effect the presence of leprosy bacilli is having on the two populations was assessed by comparing health indicators between them. The aim of these efforts was to assess whether the presence of leprosy colonisation and infection in a population has a negative effect on ERS population health.

Not all ERS populations occurring throughout the British Isles have been assessed for the presence of leprosy bacilli, including the intensely managed population around Anglesey, Wales. Thus, samples from this population were assessed for the presence of leprosy bacilli. Additionally, some samples from BI and all samples from AR were screened for both leprosy bacilli and not just the one that was expected to be present on each island. In this manner the hypothesis that both leprosy bacilli are present in ERS in different locations throughout the British Isles was challenged.

Testing of GS has been very limited (n= 4 (Avanzi *et al.*, 2016)). Thus, further active surveillance was necessary to clarify whether leprosy does affect GS in the British Isles as well as ERS. GS are the only other squirrel species established in the wild in the British Isles. They were introduced on multiple occasions in the late 19th and early 20th century (Schuchert *et al.*, 2014). They are now widespread and have almost completely replaced ERS in England, Wales, and central Scotland. Controlling GS is a vital part of ERS conservation in the UK (Mackinlay and Patterson, 2011; The Scottish Squirrel Group, 2015). GS are native to the eastern USA, where human leprosy cases do occasionally occur today. Historically, leprosy spread to the southern United States between the 16th and 18th century. First human case reports in Louisiana date back to 1758 and 1866 (International Leprosy

Association, 2019). It has been addressed as an emerging public health problem on a larger scale since 1894 (Health Resources and Services Administration, 2019). While human leprosy cases might thus have occurred occasionally in the southern parts of the natural range of GS around the time they were translocated to Europe (Shuttleworth, Lurz and Gurnell, 2016), prolonged coexistence of GS with large numbers of human leprosy patients in their natural range appears unlikely. In the British Isles human case numbers had already declined before GS arrived, but it is possible that leprosy bacilli were present in ERS populations now replaced by GS. No clinical signs of leprosy have been described in GS to date. It has not been assessed whether they are infected with leprosy bacilli in their native range.

Outside the British Isles ERS are widespread throughout their range, but at this point leprosy has not been investigated in ERS or any other wild squirrel population anywhere else. To begin to assess whether leprosy is truly limited to ERS in the British Isles, samples were acquired from two other countries within the species range, Germany and Italy. In Germany ERS are the only endemic tree squirrel species (Shar *et al.*, 2016; Wibbelt *et al.*, 2017). Other squirrel species are kept as pets though, including Southeast Asian tree squirrels (*Callosciurus spp*.). Aside from ERS the Calabrian black squirrel (*Sciurus meridionalis*) is a tree squirrel species endemic to Italy (Wauters *et al.*, 2017). Italy also has introduced wild populations of GS, Pallas's squirrels (*Callosciurus erythraeus,* PS) and Finlayson's squirrels (*Callosciurus finlaysoni,* FS). Both GS and *Callosciurus spp.* were introduced into Italy in the 1980's (Bertolino *et al.*, 2004; Martinoli *et al.*, 2010). PS are Asian tree squirrels currently listed as least concern by the IUCN. It's native range stretches from north eastern India to Myanmar, Thailand, Laos, Vietnam and south eastern China (Lurz *et al.*, 2013). FS are native to Indochina (Bertolino *et al.*, 2004). Human leprosy is still endemic to South-East Asia, with 153,487 new cases registered in 2017 alone (WHO, 2018a). For both species' exposure to human leprosy patients in their native range is thus, at least in theory, possible. However, no cases of leprosy have been reported in either species to date and introductions into Italy were limited to a few individuals which then reproduced very successfully locally (Bertolino and Lurz, 2013).

Samples of ERS (Germany, Italy), GS (Scotland, Wales, England, Italy), and PS (Italy, Germany) were screened for the presence of leprosy bacilli DNA and AFB to challenge the hypothesis that leprosy is exclusive to British ERS. While the first half of this segment of the study is largely reusing data presented in the previous chapters, the second half will present information from new sample sets and populations.

6.2. Methods

6.2.1. Leprosy in BI and AR ERS

Prevalence of leprosy in two British ERS populations

The diagnostic decision tree introduced in chapter 3 (p. 99) was used to determine the leprosy status of all ERS seen in this study on BI and AR, including both live assessed ERS and carcasses. Carcasses were included, but always treated separately from live ERS data. Including own carcass data allows a direct comparison with previously published carcass information. It also allows to compare disease occurrence in live ERS and carcasses collected in the same location and timeframe instead of only being able to compare live ERS data to carcass data in the published literature. ERS showing clinical signs of disease (i.e. cases or suspicious) were considered to be leprosy morbidity events.

For each population and session, the apparent point prevalence of clinical leprosy and non-clinical colonisation was calculated by dividing the number of ERS diagnosed as leprosy cases or colonised by the total number of ERS assessed in that sampling session, respectively. The average estimated point prevalence for live animals across all cohorts was also calculated.

Additionally, an apparent two-year prevalence estimate including each live ERS only once, was calculated for each population. For this, ERS that were diagnosed as leprosy cases or as colonised at any point within these two years were treated as cases/colonised, i.e. the maximal leprosy status a squirrel reached within the two years was used for the two-year prevalence estimate. Thus, if an ERS was first colonised but then developed clinical disease it was included as clinical case. Where ERS were identified as colonised in one session, but a later PCR had a negative result, they were still included as colonised, due to the limitations of the diagnostic tests (see chapter 3). Not all diagnostic information was available for all ERS, i.e. some tissue samples were missing. Thus, particularly the prevalence of colonisation with leprosy bacilli may be underestimated based on the current dataset.

Simple exact binominal confidence intervals for each apparent prevalence were calculated using Minitab ® 17 statistical software.

The hypothesis that there is no difference in the proportion of ERS affected by (summarising case, suspicious and colonised ERS) or diagnosed with a specific leprosy status in the two island populations was tested using a Fisher's Exact test and Wilcoxon rank sum test in R, respectively.

Incidence rate of clinical leprosy in ERS

The sample set collected in this study is not ideally suited to calculate disease incidence. For some squirrels only a single time point is available, i.e. no information whether this animal developed disease is available at all. While no ERS on AR developed clinical disease within

the study period, and incidence can thus not be calculated for this population, the 31 ERS returning multiple times on BI that were introduced in chapter 5 can be used for a first, cautious incidence estimate. To be able to attempt an incidence estimate, the assumption was made that all animals were 12 months old at the time of the first assessment. This is an artificial value and while it is likely that few animals were younger, several may have been older.

If these returning animals are treated as closed cohort, representing about 15% of the total estimated squirrel population on BI, it is possible to do a basic calculation of the incidence rate that while offering a first estimate, may be proven wrong once more data becomes available.

The incidence rate was calculated as:

$$\frac{\text{Number of disease onsets}}{\text{Sum of squirrel-time at risk}}$$

The squirrel time at risk was calculated by adding up the time for each animal of 12 months of life before the first assessment and then adding the time

a) until it was seen with lesions, in animals that did eventually develop lesions, though it is likely that lesions first occurred earlier. It was chosen to do this, as the other assumption of age at first assessment is likely to be too conservative.

b) that had passed between initial assessment and the last assessment available for an animal, in those animals that did not develop leprosy lesions within the study period.

Squirrels already diseased at the initial assessment did not contribute animal time at risk. Calculation was done twice using this squirrel time at risk, once treating BI 55, the leprosy suspect case that could not be confirmed with additional tests, as ERS that did develop clinical leprosy, and once treating this animal as not being a clinical case of leprosy. This did only influence the number of disease onsets but not the sum-of squirrel-time at risk, as the lesion in BI 55 was only observed in its final assessment.

ERS population health in the presence of *M. leprae*

Data collected on four health indicators, namely BCS, reproductive activity, GHS, and ectoparasites observed, were compared between the two populations (BI and AR). As annual and seasonal factors can influence these indicators, they were compared separately by sampling session. The influence geographic factors may have had will be addressed when discussing the results of these comparisons. No comparison was possible for autumn 2016, as only animals on BI were sampled at this time. The results for each factor in this session are still included in the graphs to illustrate how they compare to the later sessions.

A Wilcoxon rank sum test was used to test the hypothesis that there was no difference in BCS of ERS seen on the two different islands. To compare reproductive activity the breeding condition information collected during the health assessments was simplified. Male squirrels were categorised as not currently reproductively active if their testes were abdominal or scrotal without pigmentation, and as reproductively active when scrotal pigment and testes were observed. Females were categorised as currently inactive where inactivity had been noted during the health assessment and as currently reproductively active if they were in oestrus, pregnant or lactating. The fact that our trapping efforts tried to avoid times of reproductive activity does introduce an intense bias to the sample set, and it is highly likely that the apparent reproductive activity we report here is much lower than the true reproductive activity seen in these populations. As this bias was the same for both islands, it was still decided to compare this factor between the populations. It can however, not be compared to data collected from other populations at other times of the year.

For sampling sessions in which reproductively active and inactive animals were seen on both islands a Fisher's exact test was used to test the hypothesis that the proportion of reproductively active and inactive ERS is the same in both populations.

It was previously explained how a general health score was assigned to each squirrel during the health assessments or post mortem examination (p. 45). Healthy animals were either scored as in good health (1) or in good health with minor or old/healed injury (2). Acutely diseased animals were separated in those with a good prognosis (3) and those unlikely to improve (4). Chronically diseased animals were separated by whether they were able to cope with their condition (5) or not (6). A Wilcoxon rank sum test was used to test the hypothesis that there was no difference in general health of ERS seen on the two different islands.

The ranked ectoparasite intensity (0= no parasites observed; 1= less than 5 ectoparasites observed, 2= less than 10 ectoparasites observed, 3= more than 10 ectoparasites observed; see p. 43 and following) was compared between the populations to test the null hypothesis that there is no difference in intensity of infestation of ERS from the two island populations. Hypothesis testing was carried out using Wilcoxon rank sum test.

The presence of different external parasites was noted in ranks (none, ticks only, fleas only, ticks and fleas, ticks and harvest mites) and a Wilcoxon rank sum test used to test the hypothesis that there is no difference in the ectoparasites or ectoparasite combinations occurring on both islands.

6.2.2. Leprosy in other squirrel populations

Presence of leprosy bacilli in ERS from Wales

Pinnae of 61 ERS, collected for surveillance purposes from ERS found dead between 2015 and 2017, with one sample dating back to 1983, from Anglesey, Wales, were provided by a

collaborator from Bangor University. The samples were analysed for *M. leprae* and *M. lepromatosis* DNA using the protocol previously described (p. 72 and p. 108).

Presence of leprosy bacilli in GS in the United Kingdom

Adult GS had been humanely killed in pest control efforts by qualified individuals and were made available through the Scottish Wildlife Trust and Pryor & Rickett Silviculture and a collaborator at Bangor University. In assessed areas ranges of GS and ERS border on each other or overlap.

Where sufficiently intact, ears, hind and front feet had been removed by collaborators in the field and were immediately send to UoE. Some carcasses were submitted whole and ears removed during post mortem examination at UoE. Samples were separated into two groups per animal, one then immersed in formalin, the other in ethanol. Fixation time before further processing varied, but was longer than 48 h in every instance.

Samples for histological screening were processed at the UoE (RDSVS), while samples for molecular screening were transported to the Moredun Research Institute.

Tissues from 53 GS were trimmed for histological assessment. From ears a 2-3 mm wide longitudinal section presenting the full length of the ear was prepared. The section cut from feet was 2-3mm wide, longitudinal, using the most accessible oval plantar pad and allowing the section to reach into the soft skin of the sole where possible. In hind feet the hind plantar pad was also included where feasible (Figure 80).

FIGURE 80: SECTIONS OF FOOTPADS WERE ALWAYS PREPARED USING THE FOURTH TOE AND EAR SECTIONS ALWAYS CUT FROM THE CAUDAL PART OF THE EAR

Tissues from the same animal were dehydrated and embedded in the same paraffin block. Two sections per block were stained by the staff of the Easter Bush Pathology histology laboratory at RDSVS, one with ZN stain to verify the presence or absence of AFB, the other with H&E to assess the presence and nature of inflammatory reactions. The later were only to be examined when AFB were found. Slides were analysed using an Olympus BX41 light microscope mounted with an Olympus DP72 camera, using Olympus cell imaging software for Life Science Microscopy. The tissue was screened at x20 magnification, moving very slowly but continuously through the whole tissue. If suspicious structures were observed,

magnification was increased to x40 to be able to confirm or reject that a structure could be an AFB.

Tissues from the same GS described above and pinnae from the additional 24 GS were screened for the presence of *M. leprae* DNA following the same extraction and amplification protocol detailed in chapter 3 (p. 70).

Presence of leprosy bacilli in squirrels from Germany and Italy

Samples from Germany and Italy were kindly provided by collaborators at the Friedrich Loeffler Institute (Germany), Wildtierhilfe Odenwald (Germany), and the University of Milan (Italy). All samples had been collected opportunistically and banked for research purposes.

Samples were available from 65 ERS, 41 PS, and three GS. All samples available from Italy were fixed in 70% ethanol, while from Germany separate samples from most individuals were available and split into two groups as for GS and fixed in 70% ethanol and formalin, respectively (Table 26).

TABLE 26: TISSUES AVAILABLE OF ITALIAN AND GERMAN SQUIRRELS

Type of tissue	Italy (ethanol)	Germany (formalin)	Germany (ethanol)
ERS			
Pinna	43	22	22
Hind footpad	NA	22	15
Nose	NA	7	NA
PS			
Pinna	39	NA	2
GS			
Pinna	3	NA	NA

Tissues fixed in formalin were used for histological screening, while those fixed in ethanol were used for molecular analysis. Both histological screening for AFB and PCR screening for *M. leprae* and *M. lepromatosis* DNA was done following the same protocols as described for UK red squirrels in chapter 3 (p. 72). Only a limited clinical assessment of the fixed tissues for gross lesions was feasible. Additional gross pathological information was provided by the collaborators sending the samples.

6.3. Results

6.3.1. Leprosy in BI and AR ERS

Prevalence of leprosy in two British ERS populations

Based on the clinical, serological and molecular information (see appendix V for more detail, p. 236) available for each ERS seen in this study, a diagnosis following the decision tree established in chapter 3 (p. 99) was made. Results are summarised per cohort in Table 27.

TABLE 27: DIAGNOSIS MADE FOR ERS IN THE DIFFERENT SAMPLING COHORTS

Island/cohort	Leprosy case	Leprosy suspect, further tests*	Colonised	Contact (all tests done)	Contact (not all tests done)
BI					
Autumn 2016	6	1	NA	0	19
Spring 2017	8	1	6	8	3
Autumn 2017	4	0	4	6	6
Spring 2018	4	0	1	8	12
Autumn 2018	5	1	0	23	0
Carcasses	7	0	6	5	0
AR					
Spring 2017	0	0	0	17	0
Autumn 2017	0	0	0	6	0
Spring 2018	0	0	0	14	0
Autumn 2018	0	0	2	23	0
Carcasses	0	0	3	26	0

**This category was added for ERS that appear to be early cases after the clinical assessment, but could not currently be backed up with positive results in the other tests. Additional test or re-sampling at a later point in time would be necessary to clarify the status of these ERS.*

On BI the point prevalence for clinical leprosy (= morbidity) varied between 16% and 31% in the live cohorts, with an average prevalence of 21% (SD= 0.06, 95% CI= 0.14, 0.29), while the prevalence of colonisation varied between 0% and 23% with an average of 9.4% (SD= 0.11, 95% CI= -0.04, 0.23). The total affected apparent prevalence varied between 17% and 54% and was on average 30.8% (SD= 0.16, 95% CI= 0.11, 0.51). In BI carcasses the prevalence of both, clinical cases and colonisation, was higher with 39% and 33%, respectively. Thus, the total affected apparent prevalence in carcasses was 72% (95% CI= 0.47, 0.90).

On AR the prevalence of clinical leprosy was 0% in all live cohorts and in carcasses. Apparent colonisation prevalence varied between 0% and 8% in the live cohorts with an average of 2% (SD= 0.04, CI= -0.04, 0.08). The colonisation prevalence was again higher in AR carcasses than live ERS with 10% (CI= 0.02, 0.27) (Table 28, p. 177).

Over the course of this two-year study 73 individual live ERS were seen on BI and 52 on AR. These were used to estimate the apparent two-year prevalence. For BI it is 22% (95% CI= 0.13, 0.33) for clinical leprosy cases, 1% (95% CI= 0.0003, 0.074) for suspect cases and 14% (95% CI= 0.07, 0.24) for colonisation. Thus, slightly more than a third (36%/37%) of the ERS assessed in this timeframe are thought to have been affected by leprosy. For 11 out of 46 clinically negative ERS classed as contacts no PCR data was available. Therefore, up to an additional 15% of animals could in theory be colonised.

The two-year prevalence estimate for colonisation with leprosy bacilli for the ERS assessed on AR is 4% (95% CI= 0.005, 0.13). It is 0% for clinical leprosy.

TABLE 28: APPARENT PREVALENCE (AND *95%* CONFIDENCE INTERVAL) OF LEPROSY ON BI AND AR

Island/cohort	Leprosy cases (CI)	Leprosy suspect, further tests* (CI)	Colonised (CI)	Contact (all tests done)	Contact (not all tests done)
BI					
Autumn 2016	0.23 *(0.09, 0.43)*	0.04 *(0.00, 0.2)*	0 *(0.0, 0.11)*	0 *(0.0, 0.11)*	0.73 *(0.52, 0.88)*
Spring 2017	0.31 *(0.14, 0.52)*	0.04 *(0.00, 0.2)*	0.23 *(0.09, 0.43)*	0.31 *(0.14, 0.52)*	0.12 *(0.02, 0.30)*
Autumn 2017	0.2 *(0.06, 0.44)*	0 *(0.00, 0.14)*	0.2 *(0.06, 0.44)*	0.3 *(0.12, 0.54)*	0.3 *(0.12, 0.54)*
Spring 2018	0.16 *(0.05, 0.36)*	0 *(0.00, 0.11)*	0.04 *(0.001, 0.21)*	0.32 *(0.15, 0.54)*	0.48 *(0.28, 0.69)*
Autumn 2018	0.17 *(0.06, 0.36)*	0.03 *(0.001, 0.18)*	0 *(0.00, 0.098)*	0.79 *(0.60, 0.92)*	0 *(0.00, 0.098)*
Carcasses	0.39 *(0.17, 0.64)*	0 *(0.00, 0.15)*	0.33 *(0.13, 0.59)*	0.28 *(0.097, 0.53)*	0 *(0.00, 0.15)*
AR					
Spring 2017	0 *(0.00, 0.16)*	0 *(0.00, 0.16)*	0 *(0.00, 0.16)*	1 *(0.84, 1.00)*	0 *(0.00, 0.16)*
Autumn 2017	0 *(0.00, 0.39)*	0 *(0.00, 0.39)*	0 *(0.00, 0.39)*	1 *(0.61, 1.00)*	0 *(0.00, 0.39)*
Spring 2018	0 *(0.00, 0.19)*	0 *(0.00, 0.19)*	0 *(0.00, 0.19)*	1 *(0.81, 1.00)*	0 *(0.00, 0.19)*
Autumn 2018	0 *(0.00, 0.11)*	0 *(0.00, 0.11)*	0.08 *(0.01, 0.26)*	0.92 *(0.74, 0.99)*	0 *(0.00, 0.11)*
Carcasses	0 *(0.00, 0.98)*	0 *(0.00, 0.98)*	0.1 *(0.02, 0.27)*	0.9 (0.73, 0.98)	0 *(0.00, 0.98)*

Significantly more ERS are affected by leprosy on BI than on AR, both seen live (p= 0.0000065) and assessed as carcasses (p= 0.000022, Fisher's exact test). The difference remains significant when the different diagnostic outcomes (case, suspicious, colonised, contact) are considered as ranks (Wilcoxon rank sum test, p_{live}= 0.00000499), $p_{carcass}$= 0.0000076). However, when only the results from individual live trapping sessions are compared, the difference is not in all instances statistically significant, while it is always biologically relevant. In autumn 2017 and 2018 and spring 2018 the null hypothesis that there is no difference in leprosy status between ERS from the two islands cannot be rejected (p_{a17}=0.079, p_{a18}= 0.1479, p_{s18}= 0.08166). In spring 2017 it can be rejected (p_{s17}= 0.00019). When results were considered summarising ERS simply as affected (cases and colonised) or unaffected (no clinical signs and no *M. leprae* DNA isolated) by leprosy, the null hypothesis of no difference can still not be rejected for the same sessions (p_{a17}= 0.1317, p_{a18}= 0.4305, p_{s18}= 0.1392), despite clear biologically relevant differences, like the absence of leprosy affected ERS on AR in autumn 2017 and spring 2018. In autumn 2018 the proportion of affected ERS is similar for both islands, but the significant biological difference is, that no clinical disease is observed on AR while it is seen on BI. The null hypothesis of no

difference can be rejected in spring 2017 (p= 0.00015) when more of the ERS seen on BI were affected by leprosy (53.8%) than not (46.2%), while no ERS were affected by leprosy on AR.

Incidence rate of clinical leprosy in ERS

The sum of squirrel-time at risk was 618 months, or 51.5 years. Assuming BI 55 is not a clinical leprosy case the incidence rate is thus 4/51.5= 0.078. If BI 55 is indeed a clinical leprosy case, as is deemed likely, despite the negative serology (see chapter 5, p. 160 et seqq.), the incidence rate of leprosy in the BI population could be estimated to be 5/51.5 = 0.097 based on the sample set available here. This would equate to between 78 and 97 new cases of clinical leprosy in a population of 1000 squirrels (7.8 – 9.7%) per year or between ~16 and ~19 new clinical leprosy cases occurring in the ca. 200 individual strong population on BI per year.

ERS population health in the presence of *M. leprae*

In total 126 live assessments and 18 carcasses were available for BI and 62 live assessments and 29 carcasses for AR. The null hypothesis that there is no difference in body condition of ERS from the two island populations could not be rejected for live ERS in any of the sessions (spring 2017 (p= 0.55), autumn 2017 (p= 0.07), spring 2018 (p= 0.49), autumn 2018 (p= 0.15)). It was however rejected for carcasses (p= 0.008) (Figure 81). The bias present when using carcasses for comparative assessments in the two islands were already discussed in chapter 2 (p. 58). It was still decided to include the information here, as leprosy affected carcasses were present in both populations and it was of interest to see if significant differences existed that could not simply be explained by the higher proportion of road killed ERS on AR.

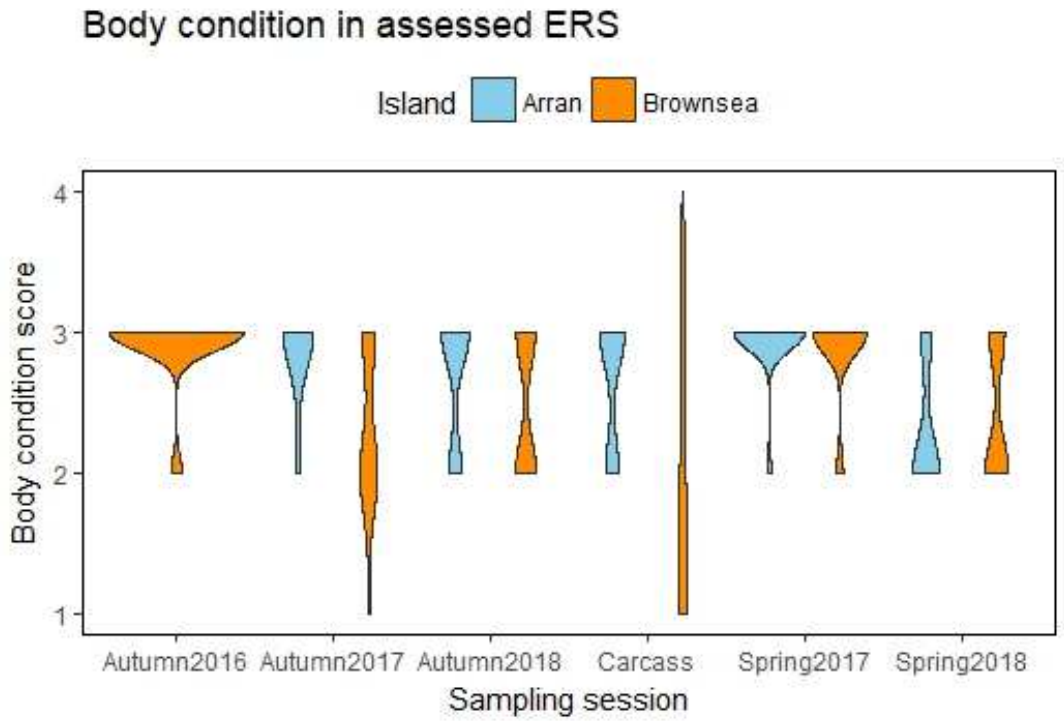


FIGURE 81: BCS OF ERS ASSESSED ON BI AND AR.

The null hypothesis that there is no difference in the proportion of reproductively active ERS in the two populations was tested for three live sampling sessions and for carcasses. As no sampling took place on AR in autumn 2016 and no reproductively active ERS were seen in autumn 2018 on either island, no hypothesis testing was possible for these sessions. For all other sessions the null hypothesis could not be rejected (Spring 2017: p= 1; Autumn 2017: p= 1; Spring 2018: p= 0.32; Carcass: p= 0.73). At least during the “off-peak” reproductive season, reproductive activity in these two populations does not show statistically significant differences (Figure 82).

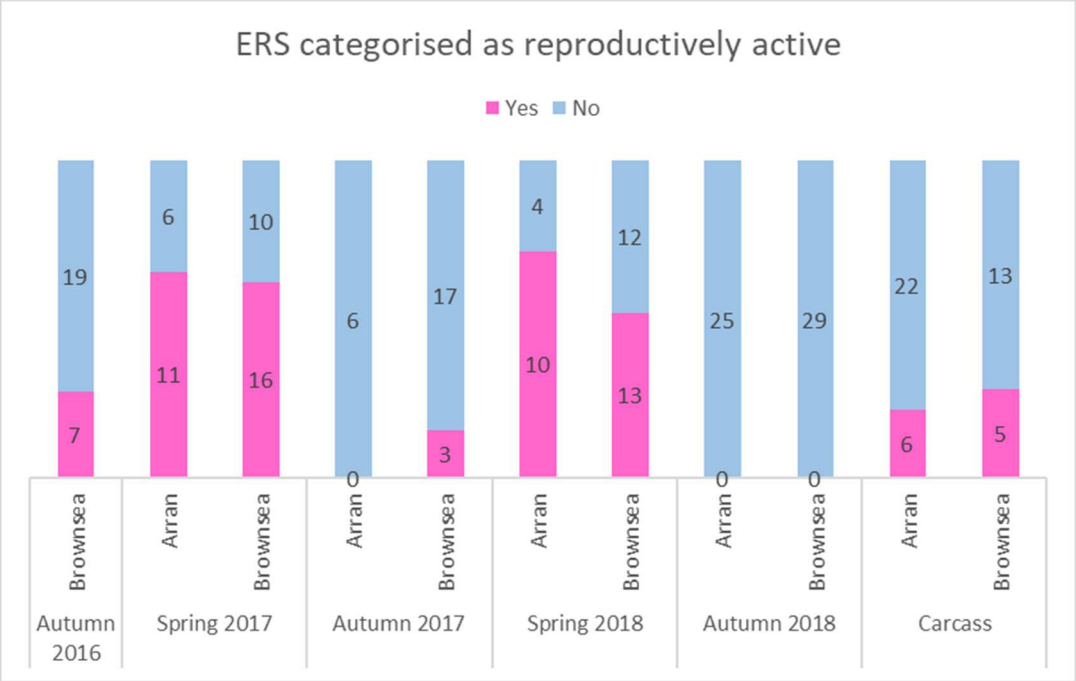


FIGURE 82: PROPORTION OF ERS CLASSED AS REPRODUCTIVELY ACTIVE IN THE DIFFERENT SAMPLING SESSIONS AND POST MORTEM ON BI AND AR. NUMBERS IN THE COLUMNS REPRESENT THE NUMBER OF ANIMALS IN A CATEGORY. PINK = REPRODUCTIVELY ACTIVE, BLUE = INACTIVE, SEXES ARE NOT SEPARATED

The null hypothesis that there is no difference in general health scores assigned in the populations could not be rejected in spring 2017 (p= 0.52), autumn 2017 (p= 0.945), autumn 2018 (p= 0.32), or carcasses collected from both islands (p= 0.77).

It was however rejected for spring 2018 (p= 0.012) (Figure 83). Overall, general health among the assessed squirrels from both islands was similar and good. Most animals seen alive were in good health (score 1 +2), only occasionally were acutely (score 3 +4) and chronically unwell (score 5 + 6) ERS seen. In carcasses acute disease unlikely to improve (score 4) was seen most often on both islands. In spring 2018 more ERS on AR showed previous injury or chronic disease than on BI.

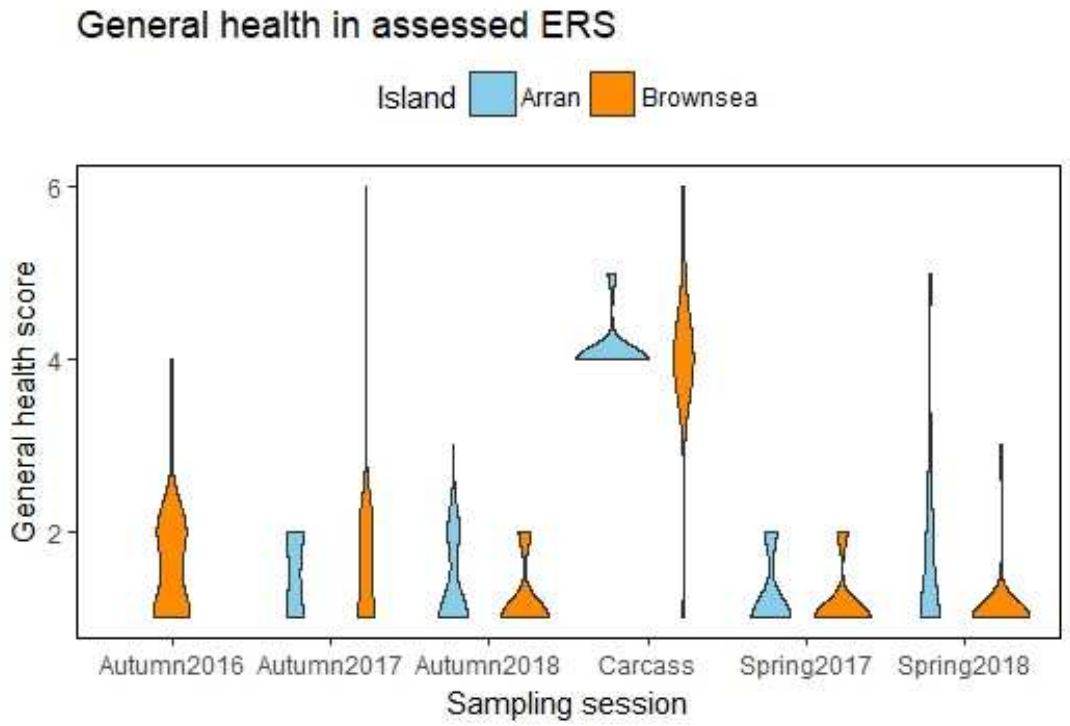


FIGURE 83: GHS (1= IN GOOD HEALTH, 2= IN GOOD HEALTH, MINOR INJURY LIKELY TO HEAL, 3= ACUTELY UNWELL, IMPROVEMENT LIKELY, 4= ACUTELY UNWELL, IMPROVEMENT UNLIKELY, 5= CHRONICALLY UNWELL, ABLE TO COPE, 6= CHRONICALLY UNWELL, UNABLE TO COPE) OF ERS ASSESSED ON BI AND AR THROUGHOUT THE TRAPPING SESSIONS

The null hypothesis that there is no difference in the intensity of ectoparasite infestation was rejected in autumn 2017 (p= 0.01), and was close to rejection in spring 2018 (p= 0.06). In both instances higher parasite intensities were seen more frequently on BI than on AR. In spring 2017 (p= 0.11) and autumn 2018 (p= 0.22) the null hypothesis could not be rejected (Figure 84).

Assessment of ectoparasite intensity was not possible from carcasses, as ectoparasites usually leave the carcass as it cools down and most carcasses were found at least hours, often days after the ERS died.

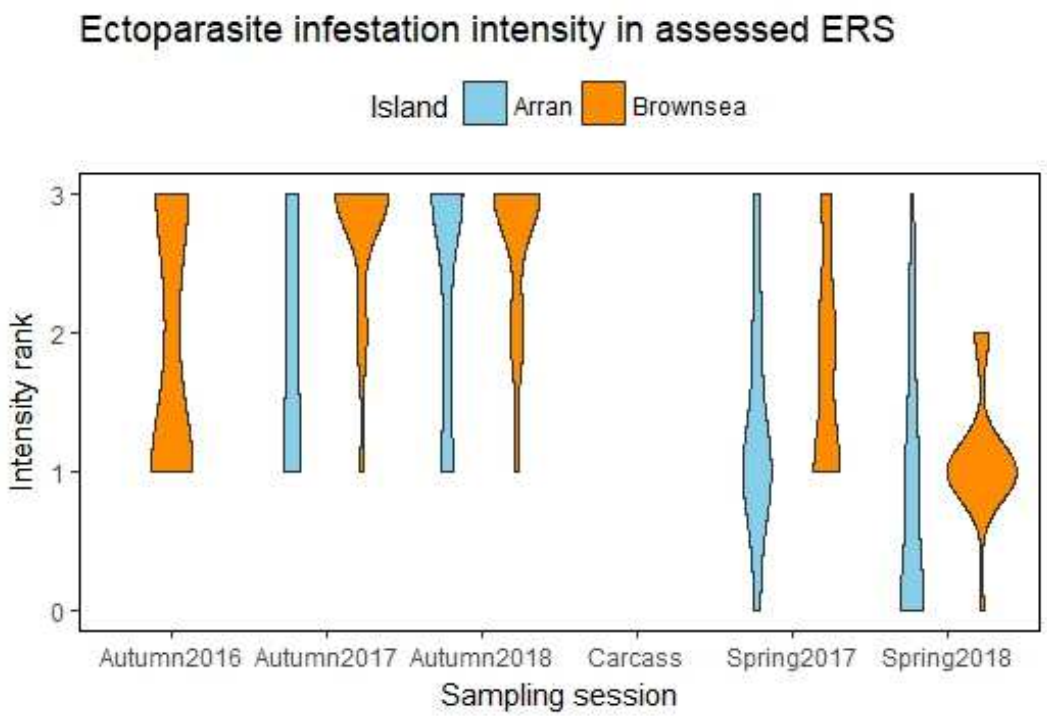


FIGURE 84: ECTOPARASITE INTENSITY FOR ERS ASSESSED LIVE ON BI AND AR. OBSERVED PARASITE LOADS ARE SLIGHTLY HIGHER ON BI, BUT THE DIFFERENCES WERE ONLY STATISTICALLY SIGNIFICANT IN AUTUMN 2017 AND SPRING 2018

In autumn 2017 (p= 0.32) and autumn 2018 (p= 0.91) the null hypothesis that there is no difference in the parasites observed on ERS during the assessments could not be rejected, despite harvest mites only being observed at this time of the year and only on BI. Most ERS seen at this time of the year were infested with ticks, and many additionally with fleas.

In spring 2017/18 (both p= 0.04) the null hypothesis was rejected. While barely any ticks appeared to be active on AR this early in the year, and some ERS here did not appear to harbour any ectoparasites at the time of trapping, ERS on BI were already infested with ticks, but these were often immature stages (Figure 85).

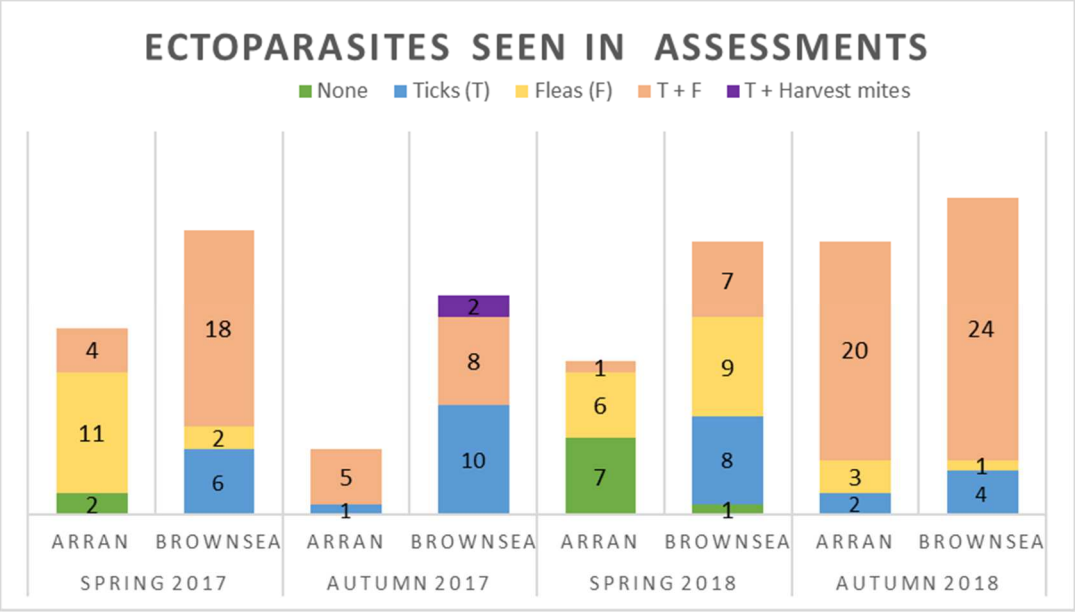


FIGURE 85: NUMBER AND TYPES OF ECTOPARASITES IDENTIFIED IN THE DIFFERENT ASSESSMENTS ON BOTH ISLANDS

Overall, none of the four health indicators suggested consistent major differences in the health of ERS from these two island populations which are affected by leprosy differently.

6.3.2. Leprosy in other squirrel populations

Presence of leprosy bacilli in ERS from Wales

No *M. lepromatosis* DNA was detected in any of the samples. *M. leprae* DNA was isolated from two samples, both collected from female squirrels in 2017. Most of the samples (n= 40) were collected in this year. These results were also published, together with results from additional screenings carried out by Charlotte Avanzi on samples from the same population, in a letter to the Veterinary Record (Schilling, Del-Pozo, *et al.*, 2019).

Presence of leprosy bacilli in GS in the United Kingdom

No AFB or *M. leprae/M. lepromatosis* DNA were present in any of the 77 GS samples tested. Samples came from Dumfries & Galloway (n= 43), the Scottish borders (n= 18), Dorchester (n= 5), and from Wales (n= 11). In these areas ranges of GS and ERS border on each other or overlap. Dorchester is an exception: here ERS are no longer present although BI is close by (ca. 9km/5.6 miles from where GS were collected). Public observations of ERS showing clinical signs indicative of leprosy have occasionally been reported from Dumfries & Galloway/the Scottish borders but not from Wales.

Presence of leprosy bacilli in squirrels from Germany and Italy

Gross lesions, AFB and *M. leprae/M. lepromatosis* DNA were absent from all samples screened from Germany and Italy. These results, together with the screening results for UK GS and additional information from samples screened by collaborators from Switzerland,

France and Mexico, were published in Frontiers of Veterinary Science (Schilling, Avanzi, *et al.*, 2019, p. xv).

6.4. Discussion

The data collected in the two focus populations of this study allowed for apparent leprosy prevalence estimates of both. Both the total prevalence and morbidity varied greatly between the populations, supporting the hypothesis that leprosy presence differs between ERS populations. While *M. leprae* DNA was successfully detected in a not previously assessed British ERS population, no leprosy bacilli were detected in the GS sampled from Britain or in any of the squirrels screened in Germany and Italy. Thus, the hypothesis that leprosy exclusively occurs in ERS in the British Isles cannot currently be refuted. All results are discussed in more detail below.

6.4.1. Leprosy in BI and AR ERS

Prevalence of leprosy in two British ERS populations

The null hypothesis that leprosy prevalence and morbidity is similar in two different British ERS populations was rejected based on the results of this study. While no leprosy related morbidity was observed on AR, 1/6 to almost 1/3 of the live ERS seen on BI in the different assessment sessions presented with clinical signs of leprosy. An even higher proportion of carcasses from this location showed clinical signs of leprosy. However, the differences in disease prevalence were not always statistically significant, as in both populations assessed here most animals were not directly affected by leprosy bacilli. They may however have had contact to leprosy bacilli through the shared environment or direct contact with colonised or diseased ERS. Additionally, results are slightly skewed by the fact that tissue samples were not taken from all ERS on BI in all sessions and thus the true proportion of affected ERS could be underestimated here, while overestimating the number of ERS just constituting contact animals.

While ERS without clinical signs of disease were found to be colonised by *M. leprae* on both islands the proportion of colonised ERS was higher on BI in most assessment sessions, despite not all ERS trapped here being tissue sampled. However, in autumn 2018, no colonised ERS were detected on BI, while two were identified on AR. The overall proportion of ERS affected by leprosy was much lower on AR than on BI.

Avanzi *et al.* (2016) had found leprosy bacilli in all BI ERS carcasses available to them (collected between 2010 and 2015), with eight out of 25 showing clinical signs of disease (32% morbidity, 100% affected). This study found a lower proportion of BI ERS affected, both when considering live sampled ERS (36/37% affected, 22/23% morbidity, two-year prevalence) and carcasses (72% affected, 39% morbidity). Even if all live ERS for which no PCR data was available in the two-year prevalence estimate were colonised, the proportion of affected ERS would at maximum be 48%. Given the variability observed in prevalence

and morbidity in the different sampling cohorts, it would be of interest to collect data over a longer period of time to assess, whether there is a variation in numbers of affected/case ERS that correlates with external changes or if differences are mainly due to chance/trapping success in the individual sessions. Generally, the data available from BI implies that there is a risk of overestimating the prevalence of ERS leprosy when only carcass data is used, making it important that live sampling is continued to be used in ERS leprosy epidemiology research.

The two-year prevalence and morbidity on BI were slightly higher than the average calculated from the individual cohorts, thus implying that individual affected/diseased ERS seen repeatedly over the course of the study might have influenced the averages. Chapter 5 had shown that leprosy is a chronic disease in ERS that can progress over at least half of an animal's expected lifespan. This may mean that tools used in chronic disease epidemiology may need to be employed to be able to for example study factors that influence leprosy development and progression in ERS. One option would be life course approaches that would involve studying the long-term effects of biological, behavioural and social factors operating across an individual's life and across generations on leprosy risk (Ben-Shlomo and Kuh, 2002).

The awareness, that carcass data may overestimate leprosy prevalence in an ERS population is important when considering data from low prevalence populations, like AR or the Isle of Wight. On AR Avanzi *et al.* (2016) had found *M. lepromatosis* DNA in one out of 10 of carcasses sampled, while this study found a 4% two-year prevalence in live ERS with no diseased ERS seen and *M. leprae* DNA in 10% (3 out of 29) of the carcasses screened. Thus, while the leprosy bacillus identified differed, the proportion of colonised carcasses was similar in this study to Avanzi *et al.* (2016). In three of the four live cohorts no leprosy bacilli DNA was detected at all. As on BI, live ERS data suggested a much lower prevalence than carcass data.

Low prevalence may result in non-detection events if ERS from a population are only sampled at one point in time or only low numbers of ERS can be assessed. On the Isle of Wight *M. lepromatosis* DNA was isolated from 1.1% (1 out of 92) of carcasses screened (Butler *et al.*, 2017), indicating that the infection may be sustained in a population at even lower rates than in the populations included in this study. This should be kept in mind when sampling sizes for prospective screening efforts are calculated. Combined with the facts that samples available from wild animals are always limited and diagnostic tests for ERS leprosy still imperfect, this makes it unlikely that true absence of leprosy from ERS populations can be determined, something that is for example possible for SQPV (Romeo *et al.*, 2019).

Unfortunately, not enough *M. leprae* DNA was available from the AR ERS to sequence the strain. It can thus not be known whether it is the same strain as found on BI or not. Little is

known regarding virulence of different strains/branches of *M. leprae*. A recent study showed that *M. leprae* strain 4P (which does not naturally occur in NBA) has a growth advantage over strain 3I (which is found in NBA in the wild) following experimental infection of NBA with one or both strains (Sharma *et al.*, 2018). This could imply that some divergence in virulence may exists between different, modern *M. leprae* strains, or that natural adaptation to a host results in lower virulence. However, it is currently thought to be unlikely that ancient and modern strains of the same lineage vary significantly in virulence based on their close genetic relatedness, since there does not appear to be a loss of any known virulence genes (Schuenemann *et al.*, 2013, 2018). Thus, differences in pathogen virulence on both islands would only be a potential explanation for the observed differences, if two separate *M. leprae* strains were present in the two populations, something that cannot be determined based on the current data. *M. lepromatosis* was not detected on AR in the current study. Based on information available from humans, clinical signs following an infection with *M. lepromatosis* can be more severe (i.e. DLL with necrotizing vasculitis) than those observed in a patient infected with *M. leprae* (Han *et al.*, 2014; Velarde-Félix, Alvarado-Villa and Vera-Cabrera, 2016; Ahuja *et al.*, 2018). However, infections with *M. lepromatosis* still appear rarer than infections with *M. leprae*, a good decade after its discovery (Ahuja *et al.*, 2018). Previous studies found that *M. leprae* and *M. lepromatosis* cause highly similar clinical signs in ERS (Avanzi *et al.*, 2016), and the fact that no further cases were identified on AR could indicate that it's virulence is at least not higher than that of *M. leprae* in ERS.

It is known that many differences exist between the two ERS populations, including their genetic composition and origins (Ballingall *et al.*, 2016; Hardouin *et al.*, 2019) as well as population densities and habitat characteristics (see chapter 2, p. 35 et seqq.). These differences could contribute to the differences in disease prevalence observed between the two islands. Studying genetically determined susceptibility of ERS populations to leprosy along with the interaction patterns of individuals within the population and with their environment could provide valuable insights into the determining factors of prevalence variation and should be addressed by future research.

The uniqueness of the current dataset and the absence of a diagnostic gold standard only allowed for an apparent prevalence estimate. Now that some initial data is available, future studies could be designed to use maximum likelihood modelling (Hui and Walter, 1980; Lewis and Torgerson, 2012) or Bayesian Estimation approaches (Joseph, Gyrokos and Coupal, 1995), to allow true prevalence estimates.

Incidence rate of clinical leprosy on BI

The proportion of diseased ERS on BI varied from session to session despite the chronic character of the disease and the good re-trapping success. While some diseased ERS were seen multiple times, disease onsets could be observed as well, implying that clinical leprosy on BI is not limited to a few ERS that are then observed again and again, but actively being

transmitted and causing new cases in the population. More long-term studies of the population would be necessary to establish whether the incidence rate estimate calculated here is truly realistic and to assess whether disease incidence is consistent over time or variable based on external influences. As mentioned above, epidemiological studies using life course approaches might be more suitable to study leprosy incidence in ERS than short-term surveillance and monitoring efforts due to the chronic nature of the disease.

ERS population health in the presence of leprosy

Live ERS on both islands were mostly in normal body condition or thin, which, as explained previously, does not necessarily mean malnourished, as substantial internal fat stores may be present. Thus, the presence of clinical leprosy and the higher rate of colonisation by leprosy bacilli on BI does not appear to put the ERS at a disadvantage where foraging and maintaining body condition is concerned. Based on the data presented in this study it appears unlikely that leprosy has a major negative impact on the nutritional status of an ERS population. Differences in BCS seen in carcasses can be explained with the high proportion of road kill animals included on AR (see chapter 2, p. 66).

There also appears to be no impact of a higher proportion of ERS in a population being affected by leprosy on reproductive success. However, this factor should further be investigated by following the populations over time and determining factors like successfully raised offspring per season as well.

There was no statistically significant difference in the GHS observed in ERS in both populations in all but one session. In this one session (spring 2018) AR ERS were in “poorer” health than the ERS on BI. However, the statistical difference observed here may not be biologically relevant. While the majority of ERS on both islands seen in spring 2018 were in good health, some of those on AR had old injuries (BI: 24 ERS GHS 1; AR: Nine ERS GHS 1, three ERS GHS 2). On each island one animal was seen with an acute injury that was likely to heal. On AR one chronically diseased animal able to cope was also seen. Therefore, the already smaller sample on AR was split across more scores than the ERS seen on BI, which influenced the statistical significance of differences.

In one out of four sessions statistically significantly higher ectoparasite intensities were observed on BI than on AR. However, this was the session in autumn 2017 during which only six ERS were trapped on AR and two of three trapping days were very rainy, when only a limited number of traps were opened to allow for more frequent checks and avoid unnecessary exposure of trapped ERS to the elements. Adverse weather conditions can have an impact on the ectoparasite burdens observed and the discrepancy in number of ERS trapped could have amplified this effect. It is thus unlikely to be related to the presence/absence of leprosy in the sampled ERS. Spring 2018 was unusually cold on both islands, still some ERS on AR had high parasite burdens, others very low. On BI most ERS

had relatively low burdens. Therefore, the statistically significant difference observed between the two populations at this time is again unlikely to be linked to the presence of leprosy bacilli in a population, as in the population with the higher parasite burdens no ERS affected by leprosy were observed.

Based on the differences in climate on the two islands (see chapter 2, p. 35 et seqq.) it could have been expected that particularly tick activity and presence on ERS would be higher on BI than on AR, as average temperatures during our sampling times were well above the threshold for activity for ticks on BI but not on AR. As for fleas, in a habitat more densely populated by ERS, drey sharing may occur more frequently and less dreys may be unused, thereby giving fleas, easier and more frequent access to a host. Thus, flea burdens could also be expected to be higher on BI than on AR, regardless of the presence of any infectious disease.

The statistically significant differences in parasite composition observed on the ERS in the spring sessions on both islands are likely due to the still much lower temperatures on AR, which would naturally reduce tick activity at this time of the year (Cardon *et al.*, 2011; Maaz *et al.*, 2018).

Thus, while the health indicators used here did flag up some differences between the two populations, these were unlikely to be linked to the differences in leprosy prevalence within the ERS populations and more likely due to differences between the two habitats, and most importantly the local climate.

The fact that the BI population, which is more intensely affected by leprosy, does not appear to be at a health disadvantage compared to the AR population allows to expand the hypothesis made for individual ERS - that the immediate impact leprosy is having is minor. Furthermore, it could now be stated that leprosy does not drive ERS population health regardless of the frequency with which it occurs in the population. This does correlate with observations in other species. In NBA a generally low impact of leprosy on wild populations is reported, regardless of the prevalence of disease (Truman, 2005; Morgan and Loughry, 2009), while hyperendemic areas of human leprosy continue to exist and do not result in mortality rates high enough to cause declines of local human populations. On the contrary, throughout the history of the disease, affected humans have - by force or voluntarily - formed colonies and lived for extended periods of time in these enclaves, sometimes having families here (White and Franco-Paredes, 2015). If the social stigma, which is only relevant to humans, is briefly ignored, negative consequences following an infection with leprosy bacilli only exist for some individuals, but in no host species for populations. Obvious disability has not been observed in ERS, as it is likely that impaired individuals would be readily predated, and loss of sensitivity was not assessed. What has occasionally been observed was pulmonary (BI 026_16) or systemic disease (BI062_17) or a large cheek abscess

(BIC002_16) in ERS also affected by leprosy. Based on these few cases it cannot be concluded though, whether leprosy was an important confounding factor in the onset of these conditions or an accidental finding. The latter could be suspected given the good health seen in the majority of affected ERS in this study.

Health indicator data collection was not the main focus for the design of the study and planning more targeted assessments in the future could be worthwhile. Particularly, these could compare the reproductive success in terms of young survival and comparing ectoparasite burdens when environmental conditions are similar for both islands, instead of comparing at the same time of the year. An additional factor that future studies might include is screening for the presence of other pathogens within the populations. This could address whether leprosy could be an indicator for weaker immunity in a population, either due to genetic make-up or over-ageing. It could also allow to assess whether being infected with leprosy increases the risk for an ERS to develop other diseases or not.

6.4.2. Leprosy in other squirrel populations

Presence of leprosy bacilli in ERS from Wales

After BI and AR Wales is now the third location in which *M. leprae* DNA has been isolated from ERS tissue samples. This illustrates that *M. leprae* is present in ERS more widely than previously reported (Avanzi *et al.*, 2016). The Welsh ERS population has undergone severe declines and genetic bottlenecks in the past, but is now actively managed towards greater diversity and external factors like habitat quality and GS are managed to favour ERS (Ogden *et al.*, 2005). This has included the successful introduction of captive bred and wild-caught ERS (Ogden *et al.*, 2005; Shuttleworth, Kenward and Jackson, 2008). It cannot be said at this point whether leprosy bacilli were introduced and transmitted as part of these re-introductions. Clinical leprosy has not been observed in any resident or introduced ERS in Wales, and based on the data from this study, bacterial levels in colonised ERS seem low. As discussed for the AR population, it cannot currently be explained how only non-clinical infection at low prevalence is being sustained in an ERS population.

Unfortunately, as for the ERS from AR, not enough *M. leprae* DNA could be extracted to sequence the Welsh strain. It is thus unknown whether the same strain is present as on BI, or if ERS here are affected by other strains that could indicate a different origin. Further efforts made in collaboration with Charlotte Avanzi, after data collection for this study was complete, to identify additional colonised ERS in the Welsh population were unsuccessful. Thus, this matter continues to be unresolved. As the Anglesey population is being closely monitored, the opportunity of continued screening for clinical signs of disease exists and it could be considered to test a proportion of all ERS carcasses available annually for the presence of leprosy bacilli to gather more information on prevalence and epidemiology over

time. However, such a measure is probably not necessary from an ERS conservation point of view.

Presence of leprosy bacilli in GS in the United Kingdom

From the information provided by Avanzi *et al.* (2016) for clinically healthy ERS in the British Isles, a case rate of 210 per 1000 was used to calculate the sample size needed to detect at least one case of leprosy with 95% confidence using Winpepi DESCRIBE. Based on this assumption a sample size of just 19 individuals should have been enough to pick up at least one GS colonised with leprosy bacilli. This number was exceeded within this study and most of our samples came from areas where there was at least some overlap with ERS identified as colonised or infected with leprosy bacilli. Thus, at least some GS included in this study are likely to have been exposed to leprosy bacilli within the shared environment. However, the data collected in this study showed that the initial assumption was too optimistic and that if the prevalence of leprosy in GS is much lower, the number of samples screened in this study may not have been large enough to identify a case. For example, if 100 colonised GS were present in every 1000, it would have already been necessary to screen 42 to find a case. This would still have been within the sample size of this study. However, if just 10 in 1000 were colonised, the necessary sample size to detect a case would increase to 446. Furthermore, there is always the risk of false negative results where bacterial loads are low. The possibility that some GS in the UK might still carry leprosy bacilli can therefore not be excluded.

GS introduced to Europe may not have had contact to leprosy bacilli in their native range, as in most of it no human or NBA leprosy cases were documented prior to the introduction of GS to Europe. In many instances it is a disadvantage to be naïve to a pathogen, as this is often linked to higher disease susceptibility. However, it is also possible that a new host is not competent to transmit a newly encountered pathogen (Daszak, Cunningham and Hyatt, 2000; Han, Kramer and Drake, 2016). Leprosy is not a highly infectious disease and leprosy bacilli are highly genetically conserved and due to their reduced genome, obligate intracellular pathogens. They need to not only successfully evade the host immune system for a successful colonisation or infection, but also find suitable conditions for reproduction in the host (Rojas-Espinosa and Lovik, 2001; Fulton *et al.*, 2016). This could make it more difficult for the pathogen to adapt to a new host species, reducing the risk of a spontaneous spill-over to a new host. Interestingly, past *M. leprae* spill-overs into a new host species seem to have occurred without obvious adaptive changes to the bacterial genome (Benjak *et al.*, 2018). Ideally future research will first clarify which genetic traits make some ERS susceptible to leprosy. It could then be assessed whether GS share these traits and are likely to be susceptible to leprosy. Apart from that only large-scale screening of hundreds of GS samples could provide further insights into the matter, but current methods would never be able to conclusively demonstrate the absence infection.

Presence of leprosy bacilli in squirrels from Germany and Italy

There are no obvious reasons why ERS in continental Europe would not have had contact to leprosy bacilli from a human source in the past. There is also the chance for more recent contacts in countries such as Italy where autochthonous transmission in humans still appears to occur (Maritati and Contini, 2016; Cusini *et al.*, 2017) or through imported leprosy cases across their range, even though these usually receive appropriate treatment quickly.

However, our sample size was relatively small, and particularly in Germany several juvenile ERS were included, a group that potentially is less likely to carry detectable levels of leprosy bacilli. Sample numbers from Italy for ERS and PS were above the minimum sample size if more than 21 in 1000 animals were affected. However, as with GS, lower prevalence is possible and, in this case, sample sizes would not have been sufficient to detect a single case. The large-scale screening efforts including hundreds of individuals necessary to detect cases if the prevalence is lower, may not appear justified for a disease that currently does not appear to pose a human or animal health threat on the European continent.

Conclusion

Data collected in the BI and AR populations showed that leprosy prevalence can vary greatly between ERS populations, even when sampling efforts and methods are comparable. Incidence could only be calculated on BI, as no morbidity events were observed on AR. Leprosy does not appear to negatively impact population health, neither in a low prevalence (AR) nor in a high prevalence population (BI), where clinical leprosy cases are regularly observed.

Detecting *M. leprae* in Wales established another location within the British Isles in which ERS are colonised or infected with leprosy bacilli. GS, on the other hand were not shown to carry leprosy bacilli, even in areas where they may at least occasionally share the environment with leprous ERS. If leprosy occurs in ERS or other squirrels in Germany and Italy, prevalence is likely to be low, based on the fact that this study did not identify any cases or colonised animals. Further studies should clarify what makes British ERS susceptible to leprosy. If susceptibility is determined by a limited set of clearly defined genetic mutations (currently no specific candidate identified, as TLR-1 results not in line with findings in other host species (Avanzi *et al.*, 2016)), it may be more practical to assess whether these traits are present in other ERS populations and squirrel species rather than to attempt large scale screenings for leprosy bacilli with diagnostic tests of limited sensitivity.

Chapter 7: Leprosy in its endangered wildlife host

7.1. Introduction

An ancient disease like leprosy can still challenge us to rethink what we believed was known about it. The recent discovery of a new pathogen species, *M. lepromatosis,* as a causative agent (Han *et al.*, 2008) and that of a completely new host species for both genetically highly conserved leprosy bacilli (Avanzi *et al.*, 2016) are two examples. However, in comparison to other mycobacterial diseases, including ones that receive much more research attention, like tuberculosis, and still pose many unanswered questions, it should perhaps not be surprising (Mukundan *et al.*, 2015).

In this study of leprosy, caused by *M. leprae* in two otherwise apparently healthy British ERS populations, methods to diagnose leprosy were adapted for use in live ERS and new insights were provided into the clinical and histological presentation as well as the clinical progression of leprosy. The study established that leprosy can be present in ERS populations at low prevalence and without causing clinical disease or may affect more than a third of the population and cause clinical disease in a high proportion of these affected animals. In both scenarios negative impacts on population health appear to be minor and negative effects on individual welfare were only observed in few, advanced cases. It was not possible to detect leprosy in GS in Britain or in squirrels in Germany or Italy. Given the relative ease and regularity with which ERS leprosy cases can be identified within the British Isles this implies that differences exist in the epidemiological situation outside the British Isles.

While this study was focussed on ERS, human interests in a potentially zoonotic pathogen that was thought to have been eradicated from the United Kingdom decades ago (Fulton *et al.*, 2016) cannot be ignored. Many are likely to agree that the main aim of mycobacterial disease research and interventions is the improvement of human health. Extensive research efforts into bovine and badger tuberculosis, for example, aim to safeguard human health and guarantee safe food. They can at the same time drive improvements in animal hygiene and welfare, and thus result in advantages for all species involved (OIE, 2019). Furthermore, appreciation that studying natural infections in animals can offer insights into drivers of host specificity, disease transmission, and comparative pathogenesis and pathology, and thus enhance the understanding of human disease, is increasing (Mukundan *et al.*, 2015). Beyond natural systems, animal models of disease are an important tool in disease research, and whether a newly discovered host could be used as such a model is a question that will inevitably be asked. This concluding discussion will thus not just focus on ERS leprosy, but also touch on what information studying leprosy in ERS can offer to human leprosy research and how.

7.2. Leprosy: a minor threat to ERS – an opportunity for research?

A case for further improvement of diagnostic methods

While this study has succeeded in adapting diagnostic methods for effective use in live ERS (chapter 3), its findings still support an important statement made by human leprosy researchers: “The absence of the diagnosis of leprosy is not the same as the absence of leprosy” (Salgado *et al.*, 2016). Even in a (diagnostic) best case in ERS where typical clinical leprosy lesions are present (chapters 3 and 4), backing up the diagnosis with laboratory methods is not always possible. While molecular methods greatly increase the ability to identify ERS colonised by leprosy bacilli, it is currently impossible to establish the true diagnostic sensitivity of such methods, as a gold standard for identifying non-clinical cases that it could be measured against does not exist. How important these non-clinical cases could be in sustaining leprosy within an ERS population is illustrated by the fact that this was the only “form” of leprosy observed on AR. While it was currently chosen to call these ERS colonised for reasons detailed in chapter 3 (p. 97), some or all of them may actually be sub- or pre-clinically infected and could play an active role in disease transmission. Developing tools to identify subclinically infected ERS is thus an important step in investigating leprosy transmission dynamics in ERS. Early diagnosis of leprosy remains a major challenge in other hosts, such as humans as well. Identifying early cellular host reactions to the presence of leprosy bacilli is seen as an important step towards achieving this (Geluk, 2018). A collaboration between human and animal immunologists could offer valuable insights in the endeavour to establish the necessary tools for early diagnosis and fuller epidemiological understanding of leprosy. While it would not immediately offer information on whether or under which circumstances an ERS will develop clinical disease, it would be a highly valuable tool in longitudinal studies investigating this matter.

The fact that the ear has proven superior to all other assessed tissues in ERS molecular leprosy diagnostics (chapter 4, p. 128) could also be of use to efforts in the human sector to increase the use of molecular diagnostic methods for early identification of cases (Salipante and Hall, 2011; Sousa Lima *et al.*, 2019). Assessing whether a similar prime diagnostic tissue exists in other hosts could be used optimise sampling strategies in surveillance programs. Currently, slit skin smears from the ear and blood samples of asymptomatic household contacts of leprosy patients are already used in human leprosy surveillance efforts (Gama *et al.*, 2018). Ensuring that these are truly the best possible samples makes sense. In NBA and other wildlife species the impact of molecular surveillance efforts could be minimized, if optimal sampling locations were to be established along with the minimum tissue volume necessary to enable diagnostics.

ERS leprosy surveillance

Why was, out of the wide range of wildlife species, leprosy discovered in endangered wild ERS? The likely answer is: “Because there was an interest by dedicated individuals and an opportunity to investigate.” Without their special status within the British Isles, it is unlikely that large scale surveillance efforts would have been made. Without dedicated researchers and, ideally, well-funded surveillance efforts covering a range of wildlife species, we are likely to continue to be unaware of the full range of host species and potential reservoirs, not only for leprosy but for many other diseases as well (Guberti, Stancampiano and Ferrari, 2014). We often simply lack the necessary knowledge about pathogen abundance and diversity in natural systems (Smith, Acevedo-Whitehouse and Pedersen, 2009). Initiatives like the PREDICT project which enables the global surveillance of pathogens that can spill over from animal hosts to people or national surveillance schemes like the APHA Diseases of Wildlife Scheme (APHA, 2019; UCDavis, 2019), are important initiatives to address these shortcomings, as are local surveillance efforts by wildlife experts and groups. They increase the ability to predict and prevent the emergence of infectious diseases. Wider conservation efforts, preserving ecosystem services including disease regulation can reduce the risk of disease spill overs (Cunningham, Daszak and Wood, 2017).

The discovery of leprosy in ERS thus underlines the importance of wildlife disease surveillance. In countries where autochthonous human leprosy cases with unknown sources are occurring, governments could consider to not just support a clinical screening of ERS whenever they are handled, but also to integrate molecular leprosy testing into wildlife surveillance efforts in general. If an animal host for leprosy does exist in these countries as well, this might allow to identify it and enable targeted follow up research as has happened here in the UK for ERS. Particularly around active leprosaria, as they exist for example in southern Europe (Suarez-Garcia *et al.*, 2017), passive surveillance in wildlife causalities might be a valuable first step in clarifying whether an animal host for leprosy bacilli is present in these countries, as exposure to human excreted pathogen is most feasible in these areas.

Leprosy lesion spectrum in ERS

This study was not able to establish whether the full spectrum of clinical and histological lesions described in other hosts are present in ERS as well. For example, TT or PNL (p. 6 and p. 10) remain undescribed in ERS. From an ERS population health and conservation perspective it is unlikely to be highly relevant whether these forms can occur. They are likely to be rare and restricted to few individuals, and in other hosts have even less short term negative effects than LL (Fischer, 2017), with which ERS can cope for extended periods of time, as seen in chapters 5 and 6 of this study. If, out of epidemiological interest, further efforts were to be made to identify such cases, this could be achieved by simply firmly adding leprosy to the differential diagnosis list for any skin lesion or peripheral neurological

impairment observed in ERS and by assessing the presence of leprosy bacilli DNA and/or AFB (ZN/FF staining) in all such cases. This could also support continued surveillance efforts in the British Isles, if they were to be made. The serological and molecular tests introduced in this study are currently not commercially available. ZN stains can however be ordered from most laboratories, and could thus provide an initial widely available screening tool for the presence of AFB in ERS carcasses, until the other methods also suitable for live ERS become widely available. Additionally, ZN staining of fine-needle aspirates from suspicious skin lesions could be a method readily accessible to many users (Fontes *et al.*, 2018), once it has been validated. As mentioned in chapter 4, it would also need to be assessed whether FF stains would be more sensitive in ERS than ZN as has been reported for other hosts (Scollard, Truman and Ebenezer, 2015). While the histological identification of AFB is straightforward and can be achieved after minimal training, full assessment and categorisation of the inflammatory reaction caused by the presence of leprosy bacilli is likely to remain a task for trained specialists, and mainly be of academic interest in ERS.

ERS leprosy transmission

The role colonised or subclinically infected individuals play in the continued transmission of leprosy still needs to be established. In humans the total number of asymptomatic patients is currently unknown, and their role in leprosy transmission thus very difficult to judge (Smith *et al.*, 2015; Neumann *et al.*, 2016). It is however assumed that clinically diseased individuals at the lepromatous/MB end of the leprosy spectrum are the main source for new leprosy infections excreting the bacteria in aerosolised form with nasal secretions (Lockwood and Suneetha, 2005; daSilva *et al.*, 2018). ERS with clinical, MB leprosy were seen on BI in this study but not on AR. MB individuals could be a source of infection for susceptible conspecifics; however, nasal discharge was not observed in any of the cases and sneezing is very rare in ERS. Furthermore, AFB were only detected in the nose of carcasses with severe, long standing clinical symptoms (chapter 4, p. 128), thus potentially limiting the time for excretion via this route to the late stages of the disease. On AR clinical cases of leprosy have not been documented in the ERS population, despite *M. leprae* and *M. lepromatosis* being present in the population. How then is the infection sustained in this population? Either the assumptions made for humans are not transferrable, and non-clinical ERS can transmit leprosy bacilli to other ERS, via a route that remains to be determined, or MB, clinically diseased ERS are present on AR and actively transmitting despite not having been observed in this study. Direct ERS to ERS contacts are mostly limited to drey sharing and interactions during mating and maternal care. Social grooming between adults is rare and intraspecies fights are generally avoided, as aggressive displays usually are enough to get another ERS to retreat (Bosch and Lurz, 2012). Other sources of infection may also exist from which members of the ERS population occasionally become infected without themselves necessarily contributing much to the further transmission of the disease while they are alive.

ERS carcasses could in theory be gnawed on by conspecifics, as particularly pregnant females are known to gnaw bones as a source of calcium (Bosch and Lurz, 2012), thus exposing other ERS to leprosy bacilli in tissues, even if their numbers remained low. However, such events are likely to be rare, in most instances ERS carcasses would be consumed by predators or scavengers. Other potential sources of infections are soil and standing water sources in the environment the ERS live in, in which leprosy bacilli can survive and remain viable for up to 45 days (Lavania *et al.*, 2008; Chinchilla, 2011; Mohanty *et al.*, 2016; Turankar *et al.*, 2016). In a pilot study in collaboration with the Geluk group at Leiden University Medical Centre screening 10 soil samples from AR and BI each did only find *M. leprae* DNA in one sample from BI and in none of the AR samples (5%) (Tio-Coma *et al.*, 2019). Not enough *M. leprae* DNA was isolated to sequence the strain and confirm that it is identical with the one present in ERS. In the same study four out of 25 soil samples (16%) collected around the dwellings of human leprosy patients in Bangladesh and three out of 28 soil samples (10.7%) collected from armadillo burrows in Suriname contained *M leprae* DNA (five species of armadillos are endemic to Suriname, including NBA). Further studies, targeting ERS caches and soil under trees with actively used dreys could help clarify whether forage, soil and water are realistic sources for leprosy infection in ERS.

Other potential sources of disease include other unknown vertebrate reservoir hosts sharing an environment with ERS, a matter that would need to be addressed by at least screening carcasses of other species found in areas to which ERS leprosy is endemic for the presence of leprosy bacilli DNA. Free living amoeba, helminths, and insects have also been implied as potentially having a role in leprosy transmission and could be relevant for sustaining infection in the ERS population as well (Blake *et al.*, 1987; Wheat *et al.*, 2014; Valois, Campos and Ignotti, 2015; Franco-Paredes and Rodriguez-Morales, 2016; Neumann *et al.*, 2016). Recent proof that *M. leprae* can be propagated in tick cell lines and that transovarial transmission of *M. leprae* occurs *in Ixodes scapularis* ticks, make it necessary to investigate the role ticks could play as vector in more depth (Ferreira *et al.*, 2018), particularly considering that at least during the warmer periods of the year ticks are regularly found on ERS.

Genetic susceptibility to leprosy in ERS

Clinically, ERS leprosy lesions can look similar to lesions described in other hosts, however, they are not identical in all characteristics, the occurrence of single nodular LL lesions being an example. It also needs to be remembered, that all clinical observations made in this study came from only one ERS population, the one on BI. They are in line with earlier observations made in ERS carcasses in Scotland and thus likely to be representative of at least part of the spectrum of lesions leprosy bacilli can cause in this host (Meredith *et al.*, 2014; Avanzi *et al.*, 2016). However, observations made in this population may not be able to tell the whole story. It is possible, that the BI *M. leprae* strain differs from the strains present on AR or Anglesey and that particular characteristics of the *M. leprae* strain present on BI are

contributing to the high proportion of clinical cases. Unfortunately, efforts to sequence *M. leprae* strains isolated from ERS on AR and in Wales failed as not enough DNA could be extracted. If the necessary pathogen samples to understand these epidemiological differences are not currently available, maybe host samples could offer some clarification. Even in the small, inbred BI population leprosy presentations differ between individuals from the apparent absence of pathogen to colonisation or clinical cases. Genetically determined host specific characteristics could be responsible for the variation within the population and for the higher proportion of clinical cases compared to other ERS populations (Simpson *et al.*, 2015; Butler *et al.*, 2017; Hardouin *et al.*, 2019). Tissue samples and DNA extracts collected throughout this study could be utilised to investigate host genetics. Avanzi *et al.* (2016) had already used a candidate gene approach to assess whether the *TLR1* gene, associated with leprosy susceptibility in humans and NBA, showed similar polymorphisms in ERS. They were not present in the expected sites. However, they did identify some mutations in this gene less frequently in leprosy infected than in healthy ERS suggesting that certain genetic traits in ERS may control their susceptibility to leprosy (Avanzi *et al.*, 2016). Since then the ERS genome has been sequenced, using samples from Britain, as part of the Wellcome Sanger Institute 25 Genomes for 25 Years project. This would allow the use of samples from BI (cases, colonised and apparently free) to identify mutations associated with different leprosy outcomes in ERS in a more open, hypothesis-free, whole genome approach, as has been proposed for humans (Cambri and Mira, 2018). Samples from ERS from other affected populations across Scotland could then be used to assess whether genetic variations identified in the inbred BI population are present in ERS susceptible to leprosy elsewhere too. If a clear genetic profile of leprosy susceptible ERS could be established, promotion of these traits could be discouraged, and where ERS are selected for breeding or translocation programs leprosy resistant ERS could be favoured.

Coinfections

An aspect that has not been covered in this study but may alter the impact leprosy is having on ERS are coinfections. The impact different co-infections may have in other hosts is not very well understood either, but appear mainly linked to alterations in the host immune response (Geluk, 2018; Sandre, Poenaru and Boggild, 2018). Currently, no coinfections with SQPV or *Staphylococcus* strains capable of causing exudative dermatitis, adenoviruses, or other infectious diseases causing mortality in ERS have been described. In this study they were not tested for. It would be important to assess the presence of such additional pathogens in ERS with different leprosy status to see if a correlation exists. Were a co-infection to be identified, that causes particularly high mortality when leprosy bacilli are also present, or amplifies the progression of leprosy, special effort would need to be made to avoid the introduction of these pathogens into ERS populations in which leprosy is present. Whether other conditions that can relate to changes in host immune response such as

pregnancy and lactation or starvation during food shortages (Lockwood and Sinha, 1999; Nogueira *et al.*, 2015; Osakunor, Sengeh and Mutapi, 2018) have an impact on leprosy progression will be difficult to study in ERS under field conditions. Individuals would have to be followed very closely and repeatedly trapped, which may create a confounding stressor.

Conservation implications of ERS leprosy

Where an interest in identifying ERS leprosy cases exists for the purpose of relocation and reintroduction purposes, it is again important to remember that the absence of a leprosy diagnosis does not equal the absence of leprosy. There is value in clinically screening ERS for leprosy lesions before entering them in translocation programs anywhere throughout their range and, in areas where leprosy is known to be endemic (i.e. the British Isles) to also use molecular screening to identify colonised ERS. Both may reduce the risk of moving ERS that may potentially be transmitting the pathogen. It will however not be possible with current methods to ensure that all ERS used in translocation efforts are free of leprosy bacilli. However, by at least avoiding translocating already chronically diseased individuals the general standards for translocation efforts would be upheld (Woodford, 2000; Mullineaux, 2018). Where ERS are intended to be released to boost an existing population of unknown leprosy status, assessing the status of the existing population as well as that of the animals to be released may be useful to at least have some level of clarity on whether leprosy was already endemic to the population. If this baseline data is available when at a later point in time cases are discovered it may be easier to decide whether a cointroduction of the pathogen has occurred. As leprosy appears to be a disease of older ERS (chapter 4, p. 135), those with clinical signs of disease are unlikely to be suitable for translocation efforts, not just because they are carrying a chronic disease, but also because they may be nearing the end of their lifespan. It can however be considered to release such individuals back to exactly where they were taken from, if individual welfare is not impaired (mild up to moderate lesions, some individuals with severe lesions but good general health and condition). Until ERS leprosy transmission is understood, little can be done beyond general good hygiene measures, including regular feeder disinfection or removal of feeders in areas where many ERS with disease symptoms are seen, to reduce disease burden in areas to which leprosy is already endemic.

Zoonotic implications

Where ERS are taken into human care, worries by individuals in contact with them, can be mitigated by attempting to diagnose leprosy in the ERS, as long as all those involved remain aware that no test can guarantee freedom of infection. In any case, good hygiene measures throughout any direct handling, and reducing handling to a minimum, something that should be standard in animals that will be released back into the wild, should mitigate the risk that may exist. The low susceptibility in the human population is likely to further ensure that the

risk of transmission even in such settings remains low (HAIRS, 2016). Where leprosy is confirmed in an ERS that has had prolonged close contact to a human, medical advice can be sought, and antibiotic post-exposure prophylaxis following WHO protocols considered (WHO, 2017). Individuals with regular contact to ERS should be aware of early symptoms of leprosy, such as anaesthetic hypopigmented skin areas, and the prolonged incubation period of the disease. Contact to ERS, and that they can be infected with leprosy bacilli in the British Isles, should explicitly be mentioned when the advice of a general practitioner is sought. This allows the general practitioner to address this concern with particular care and to rule out this possibility swiftly. A pre- and post-exposure vaccine is also under development, and may become available in the future (Duthie *et al.*, 2018). It could then become a standard vaccination for those in the British Isles working closely with ERS, just as rabies prophylaxis is a firm standard for anyone working with potential host species (Public Health England, 2018).

ERS as potential models for leprosy research

The fact, that at least nuanced differences in the clinical presentation of leprosy between ERS and other hosts exist, and that only some ERS are susceptible to the bacilli and even fewer will develop clinical disease may already limit the interest of leprosy researchers to use this host species as a model for human disease, as may the fact, that the disease, when it occurs, progresses slowly (chapter 5). With these characteristics, ERS do not immediately offer an advantage over currently established models such as NBA and mice footpads (Pena, Sharma and Truman, 2018). Only in the British Isles have ERS currently been found to be susceptible to leprosy, and here the species is under special protection (Bosch and Lurz, 2012), making it very unlikely that permission would be granted to take individuals into captivity for medical research. Additionally, the high sensitivity of ERS to stress and the high standards that enclosures need to meet for the species to thrive, make them unsuitable for laboratory experiments. While burrow dwelling NBA settle into modified rabbit laboratory cages (Pena, Sharma and Truman, 2018), i.e. into a space of roughly 60cm x 45cm x 45cm per animal (Orchid Scientific, 2019), tree-dwelling ERS will only thrive in large scale enclosures and only reproduce in captivity in optimal conditions (enclosure size ca. 7.5m x 3.7m x 3.7m for three ERS (Forder, 2006)). Thus, the cost of using captive ERS as leprosy models, even if susceptible individuals could be identified in non-protected populations, are likely to exceed the costs of existing models by far.

However, British ERS can play a role in leprosy research without being taken into captivity. In human leprosy the discussion has moved from elimination being the target of interventions to zero transmission as a new, more realistic target. It is however acknowledged that to achieve zero-transmission, transmission first needs to be understood, including all contributing factors and involved hosts and vectors (Salgado *et al.*, 2016). Naturally leprosy infected ERS could assist in understanding leprosy transmission in a ‘comparative

mycobacteriology' framework (Mukundan *et al.*, 2015). BI is a particularly valuable population in this respect, as the small island ecosystem has fewer variables to consider than most other settings in which leprosy naturally occurs, and the small, inbred population still includes three groups of ERS, those apparently resistant to leprosy, those colonised and those showing clinical signs of disease. ERS contacts on the island could be monitored, for example by using proximity collars (Mukundan *et al.*, 2015) and/or microchipping of ERS in combination with the provision of microchip reader and motion sensor camera equipped nest boxes and feeders. At the same time health status, incidence of leprosy and its progression could be monitored in regular (annual) assessments. This would provide valuable information to inform disease transmission models roughly comparable to those existing for SQPV (Tompkins, White and Boots, 2003; White *et al.*, 2016). Understanding transmission dynamics in any host species could provide transferrable information across the host range.

Integrating ancient ERS samples from museum collections into ERS leprosy research efforts could offer new clues for understanding leprosy dynamics through the ages, particularly when combined with efforts mentioned above to integrate leprosy into wildlife surveillance efforts globally.

This could become particularly relevant when considering one question: how was it possible that leprosy was apparently eliminated from the human population in the British Isles while it persisted in ERS? It is possible that non-clinical infections of humans do occur undetected. For several mycobacteria complex reservoir systems are suspected and the most important source of infection for some of these is still not determined (Haydon *et al.*, 2002; Viana *et al.*, 2014). The variety of potential sources of infection discussed by leprosy researchers, none of which, not even NBA (Schmitt *et al.*, 2010), can consistently be linked to human infection, could imply that such a complex reservoir system exists for leprosy. Ideally, future research efforts should address whether humans in the British Isles do still carry leprosy bacilli without developing clinical disease. Additionally, involving social scientists to compare the circumstances in which leprosy can be present in an animal host without human cases occurring (British Isles), with those found in areas in which leprosy is hyperendemic, ideally covering situations in which infected NBA are present and absent (i.e. Brazil and India) could be of interest. It may just underline how socio-economic conditions and hygiene appear to play major roles in reducing leprosy transmission (Schmitt *et al.*, 2010; Franco-Paredes and Rodriguez-Morales, 2016) – or it may offer new insights that have been overlooked so far, when Europe was just deemed to have generally become free of leprosy.

Concluding remarks

Leprosy appears to be an endemic disease of ERS in the British Isles. Since its identification in 2014 much has been learned, but many open questions still remain. Continued investigation of this ancient disease in its newly discovered, endangered wildlife host could

offer valuable information, and meet species conservation needs. While leprosy does not appear to pose a major conservation threat to ERS, it could hold clues to better understand disease resistance in this species. Using ERS populations in natural settings in disease research focussing on transmission dynamics could benefit humans, and may be a way to further increase the recognition of wildlife and biodiversity conservation as important contributors to safeguarding health in a One Health and conservation medicine framework.

Wordcount main text 68,002

References

Adelman, J. S. *et al.* (2015) 'Feeder use predicts both acquisition and transmission of a contagious pathogen in a north american songbird', *Proceedings of the Royal Society B: Biological Sciences*, 282(1815). doi: 10.1098/rspb.2015.1429.

Ahuja, M. *et al.* (2018) 'Detection of Mycobacterium lepromatosis in patients with leprosy in India', *Infection and Drug Resistance*, 11, pp. 1677–1683. doi: 10.1126/science.1149683.

Albert, C. H. *et al.* (2010) 'Sampling in ecology and evolution - bridging the gap between theory and practice', *Ecography*, 33(6), pp. 1028–1037. doi: 10.1111/j.1600-0587.2010.06421.x.

Anand, P. P. *et al.* (2014) 'Pretty leprosy: Another face of Hansen's disease! A review', *Egyptian Journal of Chest Diseases and Tuberculosis*. The Egyptian Society of Chest Diseases and Tuberculosis, 63(4), pp. 1087–1090. doi: 10.1016/j.ejcdt.2014.04.005.

APHA (2019) *Animal & Plant Health Agency: Wildlife disease surveillance*. Available at: http://apha.defra.gov.uk/vet-gateway/surveillance/seg/wildlife.htm (Accessed: 16 August 2019).

Araujo, S. *et al.* (2016) 'Clinical Infectious Diseases Molecular Evidence for the Aerial Route of Infection of Mycobacterium leprae and the Role of Asymptomatic Carriers in the Persistence of Leprosy', *Clinical Infectious Diseases*, 63(11), pp. 1412–1420. doi: 10.1093/cid/ciw570.

Araújo Stefani, M. M. *et al.* (2019) 'Leprosy survey among rural communities and wild armadillos from Amazonas state, Northern Brazil', *PLoS ONE*, 14(1), pp. 1–13. doi: 10.1371/journal.pone.0209491.

Aslam, S. *et al.* (2019) 'Major risk factors for leprosy in a non-endemic area of the United States: A case series', *IDCases*. Elsevier Ltd, 17, p. e00557. doi: 10.1016/j.idcr.2019.e00557.

Autorizac, D. *et al.* (2018) 'Molecular detection of Mycobacterium leprae by Polymerase Chain Reaction in captive and free-ranging wild animals', *The Brazilian Journal of Infectious Diseases*, (x x), pp. 9–11. doi: 10.1016/j.bjid.2018.09.003.

Avanzi, C. *et al.* (2016) 'Red Squirrels in the British Isles are Infected with Leprosy Bacilli', *Science*, 354(6313), pp. 744–748.

Baddam, G. S. C. *et al.* (2018) 'Validity of FNAC for the Diagnosis of Leprosy', (Dvl), pp. 137–146.

Balamayooran, G. *et al.* (2015) 'The armadillo as an animal model and reservoir host for Mycobacterium leprae', *Clinics in Dermatology*. (Leprosy: I), 33(1), pp. 108–115. doi: 10.1016/j.clindermatol.2014.07.001.

Ballingall, K. T. *et al.* (2016) 'Limited diversity associated with duplicated class II MHC-DRB genes in the red squirrel population in the United Kingdom compared with continental Europe', *Conservation Genetics*. Springer Netherlands, 17(5), pp. 1–12. doi: 10.1007/s10592-016-0852-3.

Banks, M. (1971) *A pilot study of an isolated population of red squirrels on Brownsea Island*.

Barkalow, F. S., Hamilton, R. B. and Soots, R. F. (1970) 'The Vital Statistics of an Unexploited Gray Squirrel Population', *The Journal of Wildlife Management*, 34(3), pp. 489–

500.

Barratt, E. M. *et al.* (1999) 'Genetic structure of fragmented populations of red squirrel (Sciurus vulgaris) in the UK.', *Molecular ecology*, 8(12 Suppl 1), pp. S55–S63. doi: 10.1046/j.1365-294X.1999.00771.x.

Barreto, J. G. and Salgado, C. G. (2010) 'Clinic-epidemiological evaluation of ulcers in patients with leprosy sequelae and the effect of low level laser therapy on wound healing: A randomized clinical trial', *BMC Infectious Diseases*, 10, pp. 1–9. doi: 10.1186/1471-2334-10-237.

Baumert, J. *et al.* (2014) 'A pattern of unspecific somatic symptoms as long-term premonitory signs of type 2 diabetes: Findings from the population-based MONICA/ KORA cohort study, 1984-2009', *BMC Endocrine Disorders*, 14(1), pp. 1–9. doi: 10.1186/1472-6823-14-87.

Begon, M., Townsend, C. R. and Harper, J. L. (2006) *Ecology. From Individuals to Ecosystems*. Fourth Edi. Blackwell Publishing Ltd.

Ben-Shlomo, Y. and Kuh, D. (2002) 'What is a Life Course Approach to Chronic Disease Epidemiology? Conceptual Models in Life Course Epidemiology', *International Journal of Epidemiology*, 31(Figure 2), pp. 285–293. doi: 10.1093/ije/31.2.285.

Benjak, A. *et al.* (2018) 'Phylogenomics and antimicrobial resistance of the leprosy bacillus Mycobacterium leprae', *Nature Communications*, 9(1). doi: 10.1038/s41467-017-02576-z.

Bennett, B. H., Parker, D. L. and Robson, M. (2008) 'Leprosy: steps along the journey of eradication.', *Public Health Reports*, 123(April), pp. 198–205. doi: 10.1177/003335490812300212.

Bertolino, S. *et al.* (2004) 'Activity Budget and Foraging Behavior of Introduced Callosciurus finlaysonii (Rodentia, Sciuridae) in Italy', *Journal of Mammalogy*, 85(2), pp. 254–259. doi: 10.1644/BPR-009.

Bertolino, S. and Lurz, P. W. W. (2013) 'Callosciurus squirrels: Worldwide introductions, ecological impacts and recommendations to prevent the establishment of new invasive populations', *Mammal Review*, 43(1), pp. 22–33. doi: 10.1111/j.1365-2907.2011.00204.x.

Bhat, R. M. and Prakash, C. (2012) 'Leprosy: An overview of pathophysiology', *Interdisciplinary Perspectives on Infectious Diseases*, 2012. doi: 10.1155/2012/181089.

Blackett, T. (2015) 'Squirrels - — an overview of and important diseases', *Companion animal*, 20(10), pp. 583–588.

Blackett, T. A. (2016) 'Squirrels', in Mullineaux, E. and Keeble, E. (eds) *BSAVA Manual of Wildlife Casualties*. Second. Gloucester: BSAVA, pp. 137–151.

Blackett, T. A. *et al.* (2018) 'Mortalities, amyloidosis and other diseases in free-living red squirrels (Sciurus vulgaris) on Jersey, Channel Islands', *Veterinary Record*, 183(16), p. 503. doi: 10.1136/vr.104779.

Blake, L. A. *et al.* (1987) 'Environmental Nonhuman Sources of Leprosy', *Reviews of Infectious Diseases*, 9(3), pp. 562–577.

Bobosha, K. *et al.* (2014) 'Field-Evaluation of a New Lateral Flow Assay for Detection of Cellular and Humoral Immunity against Mycobacterium leprae', *PLoS Neglected Tropical Diseases*, 8(5). doi: 10.1371/journal.pntd.0002845.

Bosch, S. and Lurz, P. W. W. (2012) *The Eurasian Red Squirrel*. First edit. Hohenwarsleben: Westarp Wissenschaften.

Bowden, S. E. and Drake, J. M. (2013) 'Ecology of Multi-host Pathogens of Animals | Learn Science at Scitable', *Nature Education Knowledge*, 4(8), p. 5. Available at: https://www.nature.com/scitable/knowledge/library/ecology-of-multi-host-pathogens-of-animals-105288915 (Accessed: 15 July 2019).

Braet, S. *et al.* (2018) 'The repetitive element RLEP is a highly specific target for detection of Mycobacterium leprae', *Journal of Clinical Microbiology*, 56(3), pp. 1–2. doi: 10.1128/JCM.01924-17.

van Brakel, W. H., Reed, N. K. and Reed, D. S. (1999) 'Grading impairment in leprosy ', *Leprosy Review* , 70(0305–7518), pp. 180–188. doi: 10.5935/0305-7518.19990021.

Bratschi, M. W. *et al.* (2015) 'Current knowledge on Mycobacterium leprae transmission: asystematic literature review', *Lepr Rev*, 86, pp. 142–155. Available at: https://www.researchgate.net/profile/Peter_Steinmann/publication/281030220_Current_knowledge_on_Mycobacterium_leprae_transmission_asystematic_literature_review_/links/55d1ace908ae502646aa5dc0.pdf.

Brodbelt, D. C. *et al.* (2008) 'The risk of death: The confidential enquiry into perioperative small animal fatalities', *Veterinary Anaesthesia and Analgesia*. Association of Veterinary Anaesthetists and American College of Veterinary Anesthesia and Analgesia, 35(5), pp. 365–373. doi: 10.1111/j.1467-2995.2008.00397.x.

Bruemmer, A. C. M. *et al.* (2010) 'Epidemiology of squirrelpox virus in grey squirrels in the UK Published by : Cambridge University Press Stable URL : http://www.jstor.org/stable/40793018 REFERENCES Linked references are available on JSTOR for this article : You may need to log in to JSTO', *Epidemiology and Infection*, 138(7), pp. 941–950.

Butler, H. M. *et al.* (2017) 'Further evidence of leprosy in Isle of Wight red squirrels.', *The Veterinary record*, 180(16), p. 407. doi: 10.1136/vr.j1920.

Cambri, G. and Mira, M. T. (2018) 'Genetic susceptibility to leprosy-from classic immune-related candidate genes to hypothesis-free, whole genome approaches', *Frontiers in Immunology*, 9(JUL), pp. 1–9. doi: 10.3389/fimmu.2018.01674.

Cardon, M. *et al.* (2011) 'Host characteristics and environmental factors differentially drive the burden and pathogenicity of an ectoparasite: A multilevel causal analysis', *Journal of Animal Ecology*, 80(3), pp. 657–667. doi: 10.1111/j.1365-2656.2011.01804.x.

Cardona-Castro, N. *et al.* (2009) 'Detection of Mycobacterium leprae DNA in nine-banded armadillos (Dasypus novemcinctus) from the Andean region of Colombia.', *Leprosy review*, 80(4), pp. 424–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20306641.

Chantrey, J. *et al.* (2014) 'European red squirrel population dynamics driven by squirrelpox at a gray squirrel invasion interface', *Ecology and Evolution*, 4(19), pp. 3788–3799. doi: 10.1002/ece3.1216.

Chilima, B. Z. *et al.* (2006) 'Distribution of Environmental Mycobacteria in Karonga District , Northern Malawi Distribution of Environmental Mycobacteria in Karonga District , Northern Malawi', *Applied and Environmental Microbiology*, 72(4), pp. 2343–2350. doi: 10.1128/AEM.72.4.2343.

Chinchilla, L. S. (2011) *Genetic variation and leprosy distribution in nine-banded armadillos*.

Mississippi State University.

Chinnadurai, S. K. *et al.* (2016) 'Best-Practice Guidelines for Field-Based Surgery and Anesthesia of Free-Ranging Wildlife. I. Anesthesia and Analgesia', *Journal of Wildlife Diseases*, 52(2s), pp. S14–S27. doi: 10.7589/52.2s.s14.

Cook, S. F. and Bies, R. R. (2016) 'Disease Progression Modeling: Key Concepts and Recent Developments', *Current Pharmacology Reports*. Current Pharmacology Reports, 2(5), pp. 221–230. doi: 10.1007/s40495-016-0066-x.

Corstjens, P. L. A. M. *et al.* (2019) 'Fingerstick test quantifying humoral and cellular biomarkers indicative for M. leprae infection', *Clinical Biochemistry*. Elsevier, (December 2018), pp. 0–1. doi: 10.1016/j.clinbiochem.2019.01.007.

Crispin, E. (1979) *A Study of the red squirrel population on Brownsea Island during the summer months*. University of Birmingham.

Cunningham, A. A., Daszak, P. and Wood, J. L. N. (2017) 'One health, emerging infectious diseases and wildlife', *Philosophical Transactions of the Royal Society of London. B.*, 372, p. 4.

Cusini, M. *et al.* (2017) 'A case of autochthonous leprosy in an elderly Italian patient leaving in Milan province with peculiar clinical presentation', *Journal of the American Academy of Dermatology*, 76(6), p. AB3. doi: 10.1016/j.jaad.2017.04.030.

daSilva, M. B. *et al.* (2018) 'Evidence of zoonotic leprosy in Para Amazon , and risks associated with human contact or consumption of armadillos', *PLoS Neglected Tropical Diseases*, 12(6). doi: e0006532.

Daszak, P., Cunningham, A. a and Hyatt, A. D. (2000) 'Emerging infectious diseases of wildlife - threats to biodiversity and human health', *Science*, 287(January), pp. 443–449. doi: 10.1126/science.287.5452.443.

Dausmann, K. H. *et al.* (2013) 'Absence of heterothermy in the European red squirrel (Sciurus vulgaris)', *Mammalian Biology*. Elsevier GmbH, 78(5), pp. 332–335. doi: 10.1016/j.mambio.2013.01.004.

Decker, D. J. *et al.* (2016) 'Wildlife Health and Public Trust Responsibilities for Wildlife Resources', *Journal of Wildlife Diseases*, 52(4), pp. 775–784. doi: 10.7589/2016-03-066.

Depoorter, P. *et al.* (2015) 'Measuring general animal health status: Development of an animal health barometer', *Preventive Veterinary Medicine*. Elsevier B.V., 118(4), pp. 341–350. doi: 10.1016/j.prevetmed.2014.12.008.

Deps, P. D. *et al.* (2008) 'Contact with armadillos increases the risk of leprosy in Brazil: a case control study ', *Indian J.Dermatol.Venereol.Leprol.* , 74(0973-3922 (Electronic)), pp. 338–342. doi: 10.4103/0378-6323.42897.

Desikan, K. and Sreevatsa (1995) 'Extendede studies on the viability of Mycobacterium leprae outside the human body.', *Leprosy Review*, (66), pp. 287–95.

Domozych, R. *et al.* (2016) 'Increasing incidence of leprosy and transmission from armadillos in Central Florida: A case series', *JAAD Case Reports*. Elsevier Inc, 2(3), pp. 189–192. doi: 10.1016/j.jdcr.2016.03.004.

Donoghue, H. D. *et al.* (2015) 'A migration-driven model for the historical spread of leprosy in medieval Eastern and Central Europe', *Infection, Genetics and Evolution*, 31, pp. 250–256.

doi: 10.1016/j.meegid.2015.02.001.

Donoghue, H. D. (2019) 'Tuberculosis and leprosy associated with historical human population movements in Europe and beyond – an overview based on mycobacterial ancient DNA', *Annals of Human Biology*. Taylor & Francis, 0(0), pp. 1–30. doi: 10.1080/03014460.2019.1624822.

DorsetWildlifeTrust (2017) *Brownsea Island Nature Reserve*. Available at: https://www.dorsetwildlifetrust.org.uk/brownsea_island_nature_reserve.html (Accessed: 10 April 2017).

Duff, J. P. *et al.* (2010) 'Wildlife: Causes of red squirrel (Sciurus vulgaris) mortality in England', *Veterinary Record*, 167(12), p. 461. doi: 10.1136/vr.c5034.

Duthie, M. S. *et al.* (2014) 'A rapid ELISA for the diagnosis of MB leprosy based on complementary detection of antibodies against a novel protein-glycolipid conjugate', *Diagnostic Microbiology and Infectious Disease*. Elsevier Inc., 79(2), pp. 233–239. doi: 10.1016/j.diagmicrobio.2014.02.006.

Duthie, M. S. *et al.* (2018) 'LepVax, a defined subunit vaccine that provides effective pre-exposure and post-exposure prophylaxis of M. leprae infection (npj Vaccines (2018) DOI: 10.1038/s41541-018-0050-z)', *npj Vaccines*. Springer US, 3(1). doi: 10.1038/s41541-018-0055-7.

Esgueva, M. F. *et al.* (2019) 'Are there Autochthonous Cases of Leprosy in Spain ? Microbial & Biochemical Technology', 11, pp. 70–72. doi: 10.4172/1948-5948.1000418.

Everest, D. J. *et al.* (2014) 'Adenovirus: An emerging factor in red squirrel Sciurus vulgaris conservation', *Mammal Review*, 44(3–4), pp. 225–233. doi: 10.1111/mam.12025.

Everest, D. J. *et al.* (2017) 'Confirmation of squirrelpox in Welsh red squirrels', *Veterinary Record*, 181(19), pp. 514–515. doi: 10.1136/vr.j5132.

Ezzedine, K. *et al.* (2009) 'Autochthonous leprosy in metropolitan France presenting with a diffuse infiltration of the face and febrile illness', *International Journal of Dermatology*, 48, pp. 69–72.

Fava, V. *et al.* (2012) 'Genetics of leprosy reactions: An overview', *Memorias do Instituto Oswaldo Cruz*, 107(SUPPL.1), pp. 132–142. doi: 10.1590/S0074-02762012000900020.

Ferreira, J. da S. *et al.* (2018) 'Ticks as potential vectors of Mycobacterium leprae: Use of tick cell lines to culture the bacilli and generate transgenic strains', *PLoS Neglected Tropical Diseases*, 12(12), pp. 1–25. doi: 10.1371/journal.pntd.0007001.

Fiegna, C. *et al.* (2016) 'Host-pathogen dynamics of squirrelpox virus infection in red squirrels (Sciurus vulgaris)', *Veterinary Microbiology*. Elsevier, 182, pp. 18–27. doi: 10.1016/j.vetmic.2015.10.012.

Fischer, M. (2017) 'Leprosy – an overview of clinical features , diagnosis , and treatment', pp. 801–827. doi: 10.1111/ddg.13301.

Fluss, R., Faraggi, D. and Reiser, B. (2005) 'Estimation of the Youden Index and its associated cutoff point', *Biometrical Journal*, 47(4), pp. 458–472. doi: 10.1002/bimj.200410135.

Fonseca, A. B. de L. *et al.* (2017) 'The influence of innate and adaptative immune responses on the differential clinical outcomes of leprosy', *Infectious Diseases of Poverty*. Infectious

Diseases of Poverty, 6(1), pp. 1–8. doi: 10.1186/s40249-016-0229-3.

Fontes, A. B. N. B. *et al.* (2018) 'Pathogen Detection', *International textbook of leprosy*, pp. 70–73. doi: 10.1136/adc.2003.042176.

Forder, V. (2006) *Captive Breeding of the Red Squirrel Sciurus vulgaris*.

Franco-Paredes, C. and Rodriguez-Morales, A. J. (2016) 'Unsolved matters in leprosy: a descriptive review and call for further research.', *Annals of clinical microbiology and antimicrobials*. BioMed Central, 15(1), p. 33. doi: 10.1186/s12941-016-0149-x.

Frota, C. C. *et al.* (2012) 'Mycobacterium leprae in six-banded (Euphractus sexcinctus) and nine-banded armadillos (Dasypus novemcinctus) in Northeast Brazil', *Memorias do Instituto Oswaldo Cruz*, 107(SUPPL.1), pp. 209–213. doi: 10.1590/S0074-02762012000900029.

Fukui, D. *et al.* (2002) 'Malignant melanoma of the eyelid in a red squirrel (Sciurus vulgaris orientis).', *The Journal of veterinary medical science / the Japanese Society of Veterinary Science*, 64(3), pp. 261–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11999447.

Fulton, N. *et al.* (2016) 'Leprosy in England and Wales 1953-2012: surveillance and challenges in low incidence countries.', *BMJ open*, 6(5), p. e010608. doi: 10.1136/bmjopen-2015-010608.

Galetti, G., Cavicchi, G. and Ussia, G. (1982) 'Replication of Mycobacterium leprae in hibernating ground squirrels (Citellus tridecemlineatus)', *Acta leprologica*, (88), pp. 23–31.

Gama, R. S. *et al.* (2018) 'High frequency of M. leprae DNA detection in asymptomatic household contacts', *BMC Infectious Diseases*, 18(1), pp. 1–6. doi: 10.1186/s12879-018-3056-2.

Gantier, M. P. *et al.* (2011) 'Analysis of microRNA turnover in mammalian cells following Dicer1 ablation', 39(13), pp. 5692–5703. doi: 10.1093/nar/gkr148.

Gardner, A. L. (2006) *ITIS Standard Report Page: Dasypodidae*, *ITIS report*. Available at: https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=180101#null (Accessed: 16 July 2019).

Gaschignard, J. *et al.* (2016) 'Pauci- and Multibacillary Leprosy: Two Distinct, Genetically Neglected Diseases', *PLoS Neglected Tropical Diseases*, 10(5), pp. 1–20. doi: 10.1371/journal.pntd.0004345.

Geluk, A. *et al.* (2012) 'New Biomarkers with Relevance to Leprosy Diagnosis Applicable in Areas Hyperendemic for Leprosy', *The Journal of Immunology*, 188(10), pp. 4782–4791. doi: 10.4049/jimmunol.1103452.

Geluk, A. (2018) 'Correlates of immune exacerbations in leprosy', *Seminars in Immunology*. Elsevier, 39(June), pp. 111–118. doi: 10.1016/j.smim.2018.06.003.

Geneva, I. I. *et al.* (2019) 'Normal Body Temperature : A Systematic Review', *Open Forum Infectious Diseases*, pp. 1–7. doi: 10.1093/ofid/ofz032.

Gill, A. L. *et al.* (2005) 'Leprosy in Britain: 50 years experience in Liverpool', *QJM - Monthly Journal of the Association of Physicians*, 98(7), pp. 505–511. doi: 10.1093/qjmed/hci079.

Gill, A. L., Gill, G. V. and Beeching, N. J. (2008) 'Familial transmission of leprosy in post-war Britain - Discrimination and dissent', *The Quarterly Journal of Medicine*, 101(5), pp. 407–413. doi: 10.1093/qjmed/hcn030.

Gilsdorf, A. and Robert Koch Institute (2017) *Infektionsepidemiologisches Jahrbuch meldepflichtiger Krankheiten für 2016.*, *Infektionsepidemiologisches Jahrbuch meldepflichtiger Krankheiten fuer 2016*. Berlin. doi: DOI: 10.17886/rkipubl-2017-002.

Gordon-Napier, G. (1933) *Some observations on the treatment of leprosy*. University of Edinburgh.

Gormus, B. J. *et al.* (1988) 'A second sooty mangabey monkey with naturally acquired leprosy: first reported possible monkey-to-monkey transmission.', *International journal of leprosy and other mycobacterial diseases : official organ of the International Leprosy Association*, 56(1), pp. 61–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3373087.

Govindan, A. *et al.* (2018) 'Immunohistochemistry of skin lesions in leprosy and leprosy reactions', *Leprosy Review*, (89), pp. 256–271.

Greco, D. and Galanti, M. R. (1983) 'Leprosy in Italy', *International journal of Leprosy*, 51(4), pp. 495–499.

Grzybowski, A., Nita, M. and Virmond, M. (2015) 'Ocular leprosy', *Clinics in Dermatology*, 33(1), pp. 79–89. doi: 10.1016/j.clindermatol.2014.07.003.

Guberti, V., Stancampiano, L. and Ferrari, N. (2014) 'Surveillance, monitoring and survey of wildlife diseases: A public health and conservation approach', *Hystrix*, 25(1), pp. 3–8. doi: 10.4404/hystrix-25.1-10114.

Gurnell, J. (2013) *Competencies for Species Survey : Red Squirrel*. Available at: www.cieem.net.

Gurung, P. *et al.* (2019) *Diagnostic accuracy of tests for leprosy: a systematic review and meta-analysis*, *Clinical Microbiology and Infection*. European Society of Clinical Microbiology and Infectious Diseases. doi: 10.1016/j.cmi.2019.05.020.

HAIRS (2016) *Human Animal Infections and Risk Surveillance (HAIRS) group Qualitative assessment of the risk that Mycobacterium lepromatosis and M. leprae in red squirrels present to the UK human population*.

Hale, M. L., Lurz, P. W. W. and Wolff, K. (2004) 'Patterns of genetic diversity in the red squirrel (Sciurus vulgaris L.): Footprints of biogeographic history and artificial introductions', *Conservation Genetics*, 5(2), pp. 167–179. doi: 10.1023/B:COGE.0000030001.86288.12.

Hamilton, H. K. *et al.* (2008) 'The role of the armadillo and sooty mangabey monkey in human leprosy', *International Journal of Dermatology*, pp. 545–550. doi: 10.1111/j.1365-4632.2008.03722.x.

Han, B. A., Kramer, A. M. and Drake, J. M. (2016) 'Global Patterns of Zoonotic Disease in Mammals', *Trends in Parasitology*. Elsevier Ltd, 32(7), pp. 565–577. doi: 10.1016/j.pt.2016.04.007.

Han, X. Y. *et al.* (2008) 'A new Mycobacterium species causing diffuse lepromatous leprosy', *American Journal of Clinical Pathology*, 130(6), pp. 856–864. doi: 10.1309/AJCPP72FJZZRRVMM.

Han, X. Y. *et al.* (2012) 'The leprosy agents Mycobacterium lepromatosis and Mycobacterium leprae in Mexico', *International Journal of Dermatology*, 51(8), pp. 952–959. doi: 10.1111/j.1365-4632.2011.05414.x.

Han, X. Y. *et al.* (2014) 'Analysis of the leprosy agents mycobacterium leprae and

mycobacterium lepromatosis in four countries', *American Journal of Clinical Pathology*, 142(4), pp. 524–532. doi: 10.1309/AJCP1GLCBE5CDZRM.

Han, X. Y. and Silva, F. J. (2014) 'On the Age of Leprosy', *PLoS Neglected Tropical Diseases*, 8(2). doi: 10.1371/journal.pntd.0002544.

Hansen, L. P., Nixon, C. M. and Havera, S. P. (1986) 'Recapture Rates and Length of Residence in an Unexploited Fox Squirrel Population', *The American Midland Naturalist*, 115(2), pp. 209–215. Available at: https://www.jstor.org/stable/2425856%0D.

Hardouin, E. A. *et al.* (2019) 'Conservation of genetic uniqueness in remaining populations of red squirrels (Sciurus vulgaris L .) in the South of England', *Ecology and Evolution*, (00), pp. 1–12. doi: 10.1002/ece3.5233.

Haydon, D. T. *et al.* (2002) 'Identifying reservoirs of infection: A conceptual and practical challenge', *Emerging Infectious Diseases*, 8(12), pp. 1468–1473. doi: 10.3201/eid0812.010317.

Health Resources and Services Administration (2019) *National Hansen's Disease (Leprosy) Program Caring and Curing Since 1894*. Available at: https://www.hrsa.gov/hansens-disease/index.html (Accessed: 24 June 2019).

Heard, D. (2014) 'Rodents', in *Zoo Animal and Wildlife Immobilization and Anaesthesia*. 2nd edn. Oxford: Wiley Blackwell, pp. 893–903.

Hobbs, H. E. *et al.* (1978) 'Ocular histopathology in animals experimentally infected with Mycobacterium leprae and M. lepraemurium. I. Mycobacterium leprae and M. lepraemurium infections in the mouse', *British Journal of Ophthalmology*, 62(8), pp. 516–524. doi: 10.1136/bjo.62.8.516.

Honap, T. P. *et al.* (2018) 'Mycobacterium leprae genomes from naturally infected nonhuman primates', *PLoS Neglected Tropical Diseases*, 12(1), pp. 1–17. doi: 10.1371/journal.pntd.0006190.

van Hooij, Anouk *et al.* (2016) 'Field-Friendly Test for Monitoring Multiple Immune Response', *BMC Infectious Diseases*. Edited by D. Scollard and T. Gillis. BMC Infectious Diseases, 8(1), p. 34260. doi: 10.1016/j.ebiom.2017.08.026.

van Hooij, A. *et al.* (2016) 'Quantitative lateral flow strip assays as User-Friendly Tools To Detect Biomarker Profiles For Leprosy', *Scientific reports*, 6(34260). doi: 10.1038/srep34260.

van Hooij, A. *et al.* (2017) 'Field-friendly serological tests for determination of M. leprae-specific antibodies.', *Scientific reports*. Springer US, 7(1), p. 8868. doi: 10.1038/s41598-017-07803-7.

van Hooij, A. *et al.* (2018) 'Evaluation of Immunodiagnostic Tests for Leprosy in Brazil, China and Ethiopia', *Scientific Reports*, 8(1), p. 17920. doi: 10.1038/s41598-018-36323-1.

Housman, G. *et al.* (2015) 'Validation of qPCR Methods for the Detection of Mycobacterium in New World Animal Reservoirs', *PLoS Neglected Tropical Diseases*, 9(11), pp. 1–13. doi: 10.1371/journal.pntd.0004198.

Hubálek, Z. and Rudolf, I. (2011) *Microbial zoonoses and sapronoses*, *Microbial Zoonoses and Sapronoses*. doi: 10.1007/978-90-481-9657-9.

Huber, M. *et al.* (2011) 'How should we define health?', *BMJ (Online)*, 343(7817), pp. 1–3. doi: 10.1136/bmj.d4163.

Hui, A. S. L. and Walter, S. D. (1980) ‘Estimating the Error Rates of Diagnostic Tests’, *Biometrics*, 36(1), pp. 167–171.

Hundeiker, M. and Broemmelhaus, H. (2007) *Wie lebten Leprakranke in Deutschland um die Wende vom 19. zum 20. Jahrhundert?*, *Die Klapper*. Available at: https://www.muenster.org/lepramuseum/07s07.pdf (Accessed: 6 November 2017).

Hutyra, F., Marek, J. and Manninger, R. (1938) *Special Pathology snd Therapeutics of the Diseases of Domestic Animals*. Fourth Eng. Edited by J. R. Greig, J. R. Mohler, and A. Eichhorn. Baillere, Tindall, and Cox, London.

ILA Technical Forum (2002) ‘Diagnosis and classification of leprosy’, *Leprosy review*, pp. 17–26. Available at: https://www.lepra.org.uk/platforms/lepra/files/lr/June02/Supplement/0006.pdf.

Inskip, S. *et al.* (2017) ‘Leprosy in pre-Norman Suffolk, UK: biomolecular and geochemical analysis of the woman from Hoxne’, *Journal of Medical Microbiology*, pp. 1–10. doi: 10.1099/jmm.0.000606.

International Leprosy Association (2019) *The United States of America | History of Leprosy*. Available at: https://leprosyhistory.org/geographical_region/country/the-united-states-of-america (Accessed: 24 June 2019).

IUCN/SSC (2013) *Guidelines for Reintroductions and Other Conservation Translocations. Version 1.0. Gland, Switzerland: IUCN Species Survival Commission*, *Ecologial Applications*.

Jamot, E. (2013) *Developing a framework for assessing wildlife health: A model for wildlife health intelligence for use in the Sri Lanka Wildlife Health Centre*.

Joseph, L., Gyrokos, T. W. and Coupal, L. (1995) ‘Bayesian estimation of disease prevalence and the parameters of diagnostic tests in the absence of a gold standard’, *American Journal of Epidemiology*, 141(3).

Jurado, F. *et al.* (2015) ‘Lucio’s leprosy: A clinical and therapeutic challenge’, *Clinics in Dermatology*. Elsevier Inc., 33(1), pp. 66–78. doi: 10.1016/j.clindermatol.2014.07.004.

Kaur, S. and Kumar, B. (1978) ‘Study of apparently uninvolved skin in leprosy as regards bacillary population at various sites’, *Leprosy in India*, 50(1), p. 38—44. Available at: http://europepmc.org/abstract/MED/349263.

Kindig, D. and Stoddart, G. (2003) ‘What Is Population Health?’, *American Journal of Public Health*, 93(3), pp. 380–383.

Kirchheimer, A. W. F. (1975) ‘Leprosy in the Nine-Banded Armadillo’, *Public Health Reports*, 90(6), pp. 483–485.

Koch, A. *et al.* (2006) ‘Leprosy: Two case reports from Dresden, Germany’, *International Journal of Dermatology*, 45(11), pp. 1321–1325. doi: 10.1111/j.1365-4632.2006.02682.x.

Kumar, A., Lambert, S. and Lockwood, D. J. (2019) *Picturing health: a new face for leprosy*, *The Lancet*. doi: 10.1016/ S0140-6736(19)30158-8.

Kunst, H. (2000) ‘Predisposing factors for recurrent skin ulcers in leprosy’, *Leprosy Review*, 71(3), pp. 363–368. doi: 10.5935/0305-7518.20000041.

Lahiri, R. *et al.* (2011) ‘Development of a mouse food pad model for detection of sub clinical leprosy.’, *Leprosy review*, 82(4), pp. 432–44. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/22439282.

Lane, J. E. *et al.* (2006) 'Borderline tuberculoid leprosy in a woman from the state of Georgia with armadillo exposure', *Journal of the American Academy of Dermatology*, 55(4), pp. 714–716. doi: 10.1016/j.jaad.2006.02.070.

LaRose, J. P. *et al.* (2010) 'Epidemiological and postmortem findings in 262 red squirrels (Sciurus vulgaris) in Scotland, 2005 to 2009.', *The Veterinary record*, 167(8), pp. 297–302. doi: 10.1136/vr.c4196.

Lastória, J. C. and de Abreu, M. A. M. M. (2014a) 'Leprosy: A review of laboratory and therapeutic aspects - Part 2', *Anais Brasileiros de Dermatologia*, 89(3), pp. 389–401. doi: 10.1590/abd1806-4841.20142460.

Lastória, J. C. and de Abreu, M. A. M. M. (2014b) 'Leprosy: Review of the epidemiological, clinical, and etiopathogenic aspects - Part 1', *Anais Brasileiros de Dermatologia*, 89(2), pp. 205–218. doi: 10.1590/abd1806-4841.20142450.

Lavania, M. *et al.* (2008) 'Detection of viable Mycobacterium leprae in soil samples: Insights into possible sources of transmission of leprosy', *Infection, Genetics and Evolution*, 8(5), pp. 627–631. doi: 10.1016/j.meegid.2008.05.007.

Levis, W., Rendini, T. and Martiniuk, F. (2018) 'Increasing Virulence in Leprosy Indicated by Global Mycobacterium spp.', *Emerging Infectious Diseases*, 24(1), pp. 183–4.

Lew, J., Yang, Y. T. and Pyun, W. S. (1974) 'Experimental infection of the Korean chipmunk (Tamias Sibiricus asiaticus, Gmelin) with M. leprae', *International Journal of Leprosy and Other Mycobacterial Diseases*, 42(2), pp. 193–202.

Lewis, F. I. and Torgerson, P. R. (2012) 'A tutorial in estimating the prevalence of disease in humans and animals in the absence of a gold standard diagnostic', *Emerging Themes in Epidemiology*, 9(1), pp. 1–8. doi: 10.1186/1742-7622-9-9.

Li, J. *et al.* (2016) 'How to improve early case detection in low endemic areas with pockets of leprosy: a study of newly detected leprosy patients in Guizhou Province, People's Republic of China', *Leprosy review*, 87(1), pp. 23–31. Available at: https://www.scopus.com/inward/record.uri?eid=2-s2.0-84976596373&partnerID=40&md5=2c4b7341225c7558c8b7057404afb3a2.

Little, L., Farmer, S. and Rosenwein, B. H. (2000) *Monks & Nuns, Saints & Outcasts: Religion in Medieval Society*. Edited by S. Farmer and B. H. Rosenwein. Ithaca, United States: Cornell University Press. Available at: http://www.cornellpress.cornell.edu/book/?GCOI=80140100332850.

Lockwood, D. N. J. and Suneetha, S. (2005) 'Leprosy: too complex a disease for a simple elimination paradigm', *Bulletin of the World Health Organization*, 83, pp. 230–235.

Lockwood, D. N. and Sinha, H. H. (1999) 'Pregnancy and leprosy: a comprehensive literature review.', *International journal of leprosy and other mycobacterial diseases : official organ of the International Leprosy Association*, 67(1), pp. 6–12.

Logas, C. M. and Holloway, K. B. (2019) 'Cutaneous leprosy in Central Florida man with significant armadillo exposure', *BMJ Case Reports*, 12(6), p. e229287. doi: 10.1136/bcr-2019-229287.

Loughry, W. J. *et al.* (2009) 'Is Leprosy Spreading Among Nine-Banded Armadillos in the Southeastern United States?', *Journal of Wildlife Diseases*, 45(1), pp. 144–152. doi:

10.7589/0090-3558-45.1.144.

Lumpkin III, L. R. *et al.* (1983) 'Leprosy in five armadillo handlers', *Journal of the American Academy of Dermatology*, 9(6), pp. 899–903. doi: http://dx.doi.org/10.1016/S0190-9622(83)70206-9.

Lurz, P. W. W. (2012) *Arran Red Squirrel Assessment 2012. Report to the Forestry Commission Scotland.*

Lurz, P. W. W. *et al.* (2013) '*Callosciurus erythraeus* (Rodentia: Sciuridae)', *Mammalian Species*, 902(902), pp. 60–74. doi: 10.1644/902.1.

Lurz, P. W. W., Gurnell, J. and Magris, L. (2005) 'Sciurus vulgaris', *Mammalian Species*, 769(769), pp. 1–10. doi: 10.1644/1545-1410(2005)769[0001:SV]2.0.CO;2.

Maaz, D. *et al.* (2018) *Factors associated with diversity, quantity and zoonotic potential of ectoparasites on urban mice and voles*, *PLoS ONE*. doi: 10.1371/journal.pone.0199385.

Mackinlay, L. and Patterson, A. (2011) *NTS Red Squirrel Conservation Action Plan*, *NTS*.

Macpherson, M. F. (2014) *Modelling population and disease dynamics in complex ecological systems*. Heriot-Watt University.

Madsen, C. L. (2011) *Population genetics of red and black squirrels in Denmark : Prospects for conservation*, *Master's thesis*. University of Copenhagen.

Malik, R., Brien, C. O. and Fyfe, J. (2009) 'Infections of cats attributable to slow growing or " non-culturable " mycobacteria'.

Mariotti, V. *et al.* (2005) 'Probable early presence of leprosy in Europe in a Celtic skeleton of the 4th-3rd century BC (Casalecchio di Reno, Bologna, Italy)', *International Journal of Osteoarchaeology*, 15(5), pp. 311–325. doi: 10.1002/oa.775.

Maritati, M. and Contini, C. (2016) 'A Case of Leprosy in Italy: A Multifaceted Disease Which Continues to Challenge Medical Doctors', *Journal of Immigrant and Minority Health*. Springer US, 18(2), pp. 490–493. doi: 10.1007/s10903-015-0223-z.

Martinez-Nicolas, A. *et al.* (2015) 'Daytime variation in ambient temperature affects skin temperatures and blood pressure: Ambulatory winter/summer comparison in healthy young women', *Physiology and Behavior*. Elsevier B.V., 149(2015), pp. 203–211. doi: 10.1016/j.physbeh.2015.06.014.

Martinez, A. N. *et al.* (2014) 'PCR-Based Techniques for Leprosy Diagnosis: From the Laboratory to the Clinic', *PLoS Neglected Tropical Diseases*, 8(4), pp. 1–8. doi: 10.1371/journal.pntd.0002655.

Martinoli, A. *et al.* (2010) 'Headcount 2010: Il proliferare delle popolazioni di scoiattolo grigio introdotte in Italia', *Hystrix*, 21(2), pp. 127–136. doi: 10.4404/Hystrix-21.2-4463.

Marzi, M. J. *et al.* (2016) 'Degradation dynamics of microRNAs revealed by a novel pulse-chase approach', pp. 554–565. doi: 10.1101/gr.198788.115.Freely.

Massone, C. *et al.* (2012) 'Imported leprosy in Italy', *Journal of the European Academy of Dermatology and Venereology*, 26(8), pp. 999–1006. doi: 10.1111/j.1468-3083.2011.04201.x.

Massone, C., Belachew, W. A. and Schettini, A. (2015) 'Histopathology of the lepromatous

skin biopsy', *Clinics in Dermatology*. Elsevier Inc., 33(1), pp. 38–45. doi: 10.1016/j.clindermatol.2014.10.003.

McDonald, K. and Larson, J. (2011) *Dasypus novemcinctus*, *Animal Diversity Web (ADW)*. Available at: https://animaldiversity.org/accounts/Dasypus_novemcinctus/ (Accessed: 18 June 2019).

McInnes, C. (2018) 'Why do red squirrels die?', *Veterinary Record*, 183(16), pp. 500–501. doi: 10.1136/vr.k4414.

McInnes, C. J. *et al.* (2009) 'First cases of squirrelpox in red squirrels (Sciurus vulgaris) in Scotland', *Veterinary Record*, 164(17), pp. 528–531. doi: 10.1136/vr.164.17.528.

Mendum, T. A. *et al.* (2014) 'Mycobacterium leprae genomes from a British medieval leprosy hospital: towards understanding an ancient epidemic', *BMC Genomics*, 15(1), p. 270. doi: 10.1186/1471-2164-15-270.

Meredith, A. *et al.* (2014) 'Leprosy in red squirrels in Scotland.', *The Veterinary record*, 175(11), pp. 285–6. doi: 10.1136/vr.g5680.

Meredith, A., Gurnell, J. and Lurz, P. (2014) *Arran Squirrel Project Final Report*.

Meredith, A. L. and Romeo, C. (2015) 'Disease and causes of mortality in red squirrel populations', in Shuttleworth, C. M., Lurz, P. W. W., and Hayward, M. W. (eds) *Red squirrels - Ecology, Conservation & Management in Europe*. European Squirrel Initiative, pp. 115–128.

Messenger, A. M., Barnes, A. N. and Gray, G. C. (2014) 'Reverse zoonotic disease transmission (Zooanthroponosis): A systematic review of seldom-documented human biological threats to animals', *PLoS ONE*, 9(2), pp. 1–9. doi: 10.1371/journal.pone.0089055.

Meteoblue (no date a) *Climate Brownsea Island - meteoblue*. Available at: https://www.meteoblue.com/en/weather/forecast/modelclimate/brownsea-island_united-kingdom_6559622 (Accessed: 23 May 2019).

Meteoblue (no date b) *Climate Isle of Arran - meteoblue*. Available at: https://www.meteoblue.com/en/weather/forecast/modelclimate/isle-of-arran_united-kingdom_2657023 (Accessed: 23 May 2019).

Meyers, W. M. *et al.* (1991) 'Naturally Acquired and Experimental Leprosy in Nonhuman-Primates', *American Journal of Tropical Medicine and Hygiene*, 44(4), pp. 24–27.

Miskin, I. P., Farrimond, P. and Head, I. M. (1999) 'Identification of novel bacterial linages as active members of microbial populations in a freshwater sediment using a rapid RNA extraction procedure and RT-PCR', *Microbiology*, 145(1 999), pp. 1977–1987. Available at: http://mic.sgmjournals.org/cgi/reprint/145/8/1977.pdf.

Mohanty, P. *et al.* (2016) 'Viability of Mycobacterium leprae in the environment and its role in leprosy dissemination', *Indian Journal of Dermatology, Venereology, and Leprology*, 82(1), p. 23. doi: 10.4103/0378-6323.168935.

Monot, M. *et al.* (2009) 'Comparative genomic and phylogeographic analysis of Mycobacterium leprae', *Nature Genetics*, 41(12), pp. 1282-U39. doi: 10.1038/ng.477.

Monte, R. S. *et al.* (2017) 'Leprosy: The Present Of A Disease From The Past', *International Archives of Medicine*, 10, pp. 1–7. doi: 10.3823/2491.

Morgan, R. E. and Loughry, W. J. (2009) ‘Consequences of exposure to leprosy in a population of wild nine-banded armadillos’, *Journal of Mammalogy*, 90(6), pp. 1363–1369. doi: Doi 10.1644/08-Mamm-a-385r.1.

Moschella, S. and Garcia-Albea, V. (2016) ‘Differential diagnosis of leprosy’, in Scollard, D. and Gillis, T. (eds) *International textbook of leprosy*.

Mukundan, H. *et al.* (2015) *Tuberculosis, leprosy and mycobacterial diseases of man and animals - The many hosts of mycobacteria*. Edited by H. Mukundan et al. Wallingford/Boston: CAB International. doi: 10.1079/9781780643960.0000.

Mullineaux, E. (2018) *Badger Rehabilitation Protocol*.

Nath, I., Saini, C. and Valluri, V. L. (2015) ‘Immunology of leprosy and diagnostic challenges’, *Clinics in Dermatology*. Elsevier Inc., 33(1), pp. 90–98. doi: 10.1016/j.clindermatol.2014.07.005.

NationalTrust (2017a) *Brownsea Island | National Trust*. Available at: https://www.nationaltrust.org.uk/brownsea-island (Accessed: 10 April 2017).

NationalTrust (2017b) *Brownsea Island red squirrels | National Trust*. Available at: https://www.nationaltrust.org.uk/brownsea-island/features/red-squirrels-on-brownsea-island (Accessed: 10 April 2017).

NationalTrust (2017c) *The history of Brownsea Island | National Trust*. Available at: https://www.nationaltrust.org.uk/brownsea-island/features/the-history-of-brownsea-island (Accessed: 10 April 2017).

NationalTrust (2017d) *Woodland wildlife and Coastal creatures | National Trust*. Available at: https://www.nationaltrust.org.uk/brownsea-island/features/other-habitats-and-wildlife-to-spot-on-brownsea-island (Accessed: 10 April 2017).

Neumann, A. da S. *et al.* (2016) ‘Experimental Infection of Rhodnius prolixus (Hemiptera, Triatominae) with Mycobacterium leprae Indicates Potential for Leprosy Transmission.’, *PloS one*, 11(5), p. e0156037. doi: 10.1371/journal.pone.0156037.

Nogueira, P. *et al.* (2015) ‘Characteristics of pregnant and lactating women with leprosy.’, *Revista Da Sociedade Brasileira De Medicina Tropical*, 48(1), pp. 96–98. doi: http://dx.doi.org/10.1590/0037-8682-0148-2014.

Ogden, R. *et al.* (2005) ‘Genetic management of the red squirrel, Sciurus vulgaris: A practical approach to regional conservation’, *Conservation Genetics*, 6(4), pp. 511–525. doi: 10.1007/s10592-005-9006-8.

OIE (2019) *Bovine tuberculosis*, *Animal Health in the World*. Available at: https://www.oie.int/en/animal-health-in-the-world/animal-diseases/bovine-tuberculosis/ (Accessed: 16 August 2019).

Oli, M. K. *et al.* (2017) ‘Dyanmics of leprosy in nine-banded armadillos: Net reproductive number and effects on host population dynamics’, *Ecological Modelling*. Elsevier B.V., 350, pp. 100–108. doi: 10.1016/j.ecolmodel.2017.02.001.

Orchid Scientific (2019) *Rabbit Cage Standard and Trolley*. Available at: https://orchidscientific.com/product/rabbit-cage-standard-trolley/ (Accessed: 31 July 2019).

Osakunor, D. N. M., Sengeh, D. M. and Mutapi, F. (2018) ‘Coinfections and comorbidities in African health systems: At the interface of infectious and noninfectious diseases’, *PLoS*

Neglected Tropical Diseases, 12(9), pp. 1–20. doi: 10.1371/journal.pntd.0006711.

Paige, C. F., Scholl, D. T. and Truman, R. W. (2002) 'Prevalence and incidence density of Mycobacterium leprae and Trypanosoma cruzi infections within a population of wild nine-banded armadillos', *American Journal of Tropical Medicine and Hygiene*, 67(5), pp. 528–532.

Palmer, S. R. (2011) *Oxford textbook of zoonoses: biology, clinical practice, and public health control*. Second. Edited by S. R. Palmer, Soulsby, and D. I. H. Simpson. New York: Oxford University Press.

Papadimitriou, D. *et al.* (2008) 'The use of mice and rats as animal models for cardiopulmonary resuscitation research', *Laboratory Animals*, 42(3), pp. 265–276. doi: 10.1258/la.2007.006035.

Parker, W. T. *et al.* (2008) 'Field Use of Isoflurane for Safe Squirrel and Woodrat Anesthesia', *Journal of Wildlife Management*, 72(5), pp. 1262–1266. doi: 10.2193/2007-141.

Patel, L. (2008) 'Growth and chronic disease', *Annales Nestle*, 65(3), pp. 129–136. doi: 10.1159/000112235.

Pedrini, S. C. B. *et al.* (2010) 'Search for Mycobacterium leprae in wild mammals', *The Brazilian Journal of Infectious Diseases*. Elsevier, 14(1), pp. 47–53. doi: 10.1016/S1413-8670(10)70010-6.

Pena, M. T., Sharma, R. and Truman, R. W. (2018) 'The Armadillo Model for Leprosy', in Scollard, D. M. and Gillis T P (eds) *International textbook of leprosy*. Available at: http://www.internationaltextbookofleprosy.org/chapter/armadillos?sect=chapter.

Perret, J.-L. *et al.* (2000) 'Influence of saturation deficit and temperature on Ixodes ricinus tick', *Parasitol Res*, 86(2000), pp. 554–557. Available at: https://link.springer.com/content/pdf/10.1007%2Fs004360000209.pdf.

Di Pierro, E. *et al.* (2010) 'Estimating offspring production using capture-mark-recapture and genetic methods in red squirrels', *Ecological Research*, 25(2), pp. 395–402. doi: 10.1007/s11284-009-0667-5.

Pin, D. *et al.* (2014) 'Species Related from Cows with Bovine Nodular Thelitis', 20(12), pp. 2111–2114.

Pinheiro, R. O. *et al.* (2018) 'Innate immune responses in leprosy', *Frontiers in Immunology*, 9(MAR), pp. 1–15. doi: 10.3389/fimmu.2018.00518.

Polycarpou, A., Walker, S. L. and Lockwood, D. N. (2013) 'New findings in the pathogenesis of leprosy and implications for the management of leprosy.', *Current Opinion in Infectious Diseases*, 26, pp. 413–419. doi: 10.1097/QCO.0b013e3283638b04.

Public Health England (2018) *Rabies: the green book, chapter 27*, *The Green Book*. Available at: https://www.gov.uk/government/publications/rabies-the-green-book-chapter-27 (Accessed: 31 July 2019).

Purtilo, D. *et al.* (1974) 'Impact of cool temperatures on transformation of human and armadillo lymphocytes (Dasypus novemcinctus,Linn.) as related to leprosy', *Nature*, 248(5447), pp. 450–452. doi: 10.1038/248450a0.

Quao, B. O. and Amankrah-Otabir, E. (2016) *Leprosy. In: Neglected Tropical Diseases - Sub-Saharan Africa*, *Neglected Tropical Diseases-Sub-Saharan Africa*. Edited by J.

Gyapong and B. Boatin. Springer.

Rafi, M. A. and Alavi, A. (2017) ‘Debate on human aging and lifespan’, *Tabriz University of Medical Sciences*, 7(3), pp. 135–137. doi: 10.15171/bi.2017.16.

Ramos, J. M., Romero, D. and Belinchón, I. (2016) ‘Epidemiology of Leprosy in Spain: The Role of the International Migration’, *PLoS Neglected Tropical Diseases*, 10(3), pp. 1–13. doi: 10.1371/journal.pntd.0004321.

Rao, P. N. *et al.* (2005) ‘Comparison of two systems of classification of leprosy based on number of skin lesions and number of body areas involved--a clinicopathological concordance study.’, *Indian journal of dermatology, venereology and leprology*, 71(1), pp. 14–19. doi: 10.4103/0378-6323.13779.

Rastogi, N., Legrand, E. and Sola, C. (2001) ‘The mycobacteria: an introduction to nomenclature and pathogenesis.’, *Revue scientifique et technique (International Office of Epizootics)*, 20(1), pp. 21–54. doi: 10.20506/rst.20.1.1265.

Rathinam, S. R., Khazaei, H. M. and Job, C. K. (2008) ‘Histopathological study of ocular erythema nodosum leprosum and post-therapeutic scleral perforation: A case report ’, *Indian J.Ophthalmol.* , 56(0301-4738 (Print)), pp. 417–419. doi: 10.4103/0301-4738.42421.

Real, L. A. (1996) ‘Sustainability and the Ecology of Infectious Disease’, *BioScience*, 46(2), pp. 88–97. doi: 10.2307/1312811.

Reibel, F. *et al.* (2015) ‘New Insights into the Geographic Distribution of Mycobacterium leprae SNP Genotypes Determined for Isolates from Leprosy Cases Diagnosed in Metropolitan France and French Territories’, *PLoS Neglected Tropical Diseases*, 9(10), pp. 1–10. doi: 10.1371/journal.pntd.0004141.

Reibel, F., Cambau, E. and Aubry, A. (2015) ‘Update on the epidemiology, diagnosis, and treatment of leprosy’, *Medecine et Maladies Infectieuses*. Elsevier Masson SAS, 45(9), pp. 383–393. doi: 10.1016/j.medmal.2015.09.002.

Rendini, T. and Levis, W. (2017) ‘Authochthonous Leprosy without Armadillo Exposure, Eastern United States’, *Emerging Infectious Diseases*, 23(11), p. 1928. doi: DOI: https://doi.org/10.3201/eid2311.171145.

Ridley, D. S. and Jopling, W. H. (1966) ‘Classification of Leprosy According to Immunity’, *International Journal of Leprosy*, 34(3), pp. 255–273. doi: 10.1126/science.1238286.

Robertson, J. (2008) ‘Leprosy, Historical’, *International Encyclopedia of Public Health*. Academic Press, pp. 65–74. doi: 10.1016/B978-012373960-5.00373-7.

Rojas-Espinosa, O. and Lovik, M. (2001) ‘Mycobacterium leprae and Mycobacterium lepraemurium infections in domestic and wild animals’, *Rev.Sci.Tech.*, 20(1), pp. 219–251.

Romeo, C. *et al.* (2019) ‘Disease, invasions and conservation: no evidence of squirrelpox virus in grey squirrels introduced to Italy’, *Animal Conservation*, 22(1), pp. 14–23. doi: 10.1111/acv.12433.

Rushton, S. P. *et al.* (2000) ‘Modelling the Spatial Dynamics of Parapoxvirus Disease in Red and Grey Squirrels : A Possible Cause of the Decline in the Red Squirrel in the UK ? Author (s): S . P . Rushton , P . W . W . Lurz , J . Gurnell , R . Fuller Published by : British Ecologica’, *Journal of Applied Ecology*, 37(6), pp. 997–1012.

Rushton, S. P. *et al.* (2006) ‘Disease threats posed by alien species: The role of a poxvirus

in the decline of the native red squirrel in Britain', *Epidemiology and Infection*, 134(3), pp. 521–533. doi: 10.1017/S0950268805005303.

Sakaguchi, M. (2009) 'Differences between body condition scores and body weight changes in postpartum dairy cows in relation to parity and reproductive indices', *Canadian Veterinary Journal*, 50(6), pp. 649–653.

Salgado, C. G. *et al.* (2016) 'What do we actually know about leprosy worldwide?', *The Lancet Infectious Diseases*. Elsevier Ltd, 16(7), p. 778. doi: 10.1016/S1473-3099(16)30090-1.

Salgado, C. G., Barreto, J. G., *et al.* (2018) 'Are leprosy case numbers reliable?', *The Lancet Infectious Diseases*, 18(2), pp. 135–137. doi: 10.1016/S1473-3099(18)30012-4.

Salgado, C. G., Pinto, P., *et al.* (2018) 'miRNome expression analysis reveals new players on leprosy immune physiopathology', *Frontiers in Immunology*, 9(MAR), pp. 1–17. doi: 10.3389/fimmu.2018.00463.

Salipante, S. J. and Hall, B. G. (2011) 'Towards the molecular epidemiology of Mycobacterium leprae: Strategies, successes, and shortcomings', *Infection, Genetics and Evolution*. Elsevier B.V., 11(7), pp. 1505–1513. doi: 10.1016/j.meegid.2011.06.003.

Sandre, M. K., Poenaru, S. M. and Boggild, A. K. (2018) 'Erythema Nodosum Leprosum Triggered by Antecedent Influenza Vaccine and Respiratory Tract Infection: A Case Report', *Journal of Cutaneous Medicine and Surgery*, p. 120347541880875. doi: 10.1177/1203475418808759.

de Santana, J. F. *et al.* (2018) 'Engineered biomarkers for leprosy diagnosis using labeled and label-free analysis', *Talanta*. Elsevier B.V., 187(March), pp. 165–171. doi: 10.1016/j.talanta.2018.05.027.

Saunderson, P. R. (2008) 'Leprosy elimination: not as straightforward as it seemed.', *Public health reports (Washington, D.C. : 1974)*, 123(2), pp. 213–6. doi: 10.1177/003335490812300214.

Schilling, A.-K., Avanzi, C., *et al.* (2019) 'British Red Squirrels Remain the Only Known Wild Rodent Host for Leprosy Bacilli', *Frontiers in Veterinary Science*, 6(February), pp. 6–11. doi: 10.3389/fvets.2019.00008.

Schilling, A.-K., Hooij, A. Van, *et al.* (2019) 'Detection of humoral immunity to mycobacteria causing leprosy in Eurasian red squirrels (Sciurus vulgaris) using a quantitative rapid test', *European Journal of Wildlife Research*. European Journal of Wildlife Research, 65(49). doi: 10.1007/s10344-019-1287-1.

Schilling, A.-K., Del-Pozo, J., *et al.* (2019) 'Leprosy in red squirrels in the UK', *Veterinary Record*, 184(13), pp. 416.1-416. doi: 10.1136/vr.l1385.

Schmitt, J. V. *et al.* (2010) 'Armadillo meat intake was not associated with leprosy in a case control study, Curitiba (Brazil)', *Memorias do Instituto Oswaldo Cruz*, 105(7), pp. 857–862. doi: S0074-02762010000700003 [pii].

Schuchert, P. *et al.* (2014) 'Landscape scale impacts of culling upon a European grey squirrel population: can trapping reduce population size and decrease the threat of squirrelpox virus infection for the native red squirrel?', *Biological Invasions*, 16(11), pp. 2381–2391. doi: 10.1007/s10530-014-0671-8.

Schuenemann, V. J. *et al.* (2013) 'Genome-wide comparison of medieval and modern

Mycobacterium leprae', *Science*. American Association for the Advancement of Science, 341(6142), pp. 179–183. doi: 10.1126/science.1238286.

Schuenemann, V. J. *et al.* (2018) 'Ancient genomes reveal a high diversity of Mycobacterium leprae in medieval Europe', *PLOS Pathogens*, 14(5), p. e1006997. doi: 10.1371/journal.ppat.1006997.

Scollard, D. M. *et al.* (2006) 'The continuing challenges of leprosy', *Clinical Microbiology Reviews*, 19(2), pp. 338–381. doi: 10.1128/CMR.19.2.338-381.2006.

Scollard, D. M. (2016) 'Editorial Infection with Mycobacterium lepromatosis', 95(3), pp. 500–501. doi: 10.4269/ajtmh.16-0473.

Scollard, D. M., Truman, R. W. and Ebenezer, G. J. (2015) 'Mechanisms of nerve injury in leprosy', *Clinics in Dermatology*. Elsevier B.V., 33(1), pp. 46–54. doi: 10.1016/j.clindermatol.2014.07.008.

Shar, S. *et al.* (2016) *Sciurus vulgaris*, *The IUCN Red List of Threatened Species*. The IUCN Red List of Threatened Species. Available at: http://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T20025A22245887.en. (Accessed: 20 August 2018).

Sharma, R. *et al.* (2013) 'The armadillo: a model for the neuropathy of leprosy and potentially other neurodegenerative diseases.', *Disease models & mechanisms*, 6(1), pp. 19–24. doi: 10.1242/dmm.010215.

Sharma, R. *et al.* (2015) 'Zoonotic leprosy in the southeastern United States', *Emerging Infectious Diseases*, 21(12), pp. 2127–2134. doi: 10.3201/eid2112.150501.

Sharma, R. *et al.* (2018) 'Differential growth of Mycobacterium leprae strains (SNP genotypes) in armadillos', *Infection, Genetics and Evolution*, 62(April), pp. 20–26. doi: 10.1016/j.meegid.2018.04.017.

Shuttleworth, C., Lurz, P. W. W. and Gurnell, J. (2016) *The Grey Squirrel: Ecology & Management of an Invasive Species in Europe 2016*. Edited by C. Shuttleworth, P. Lurz, and J. Gurnell. European Squirrel Initiative.

Shuttleworth, C. M. *et al.* (2015) 'Assessing causes and significance of red squirrel (Sciurus vulgaris) mortality during regional population restoration: An applied conservation perspective', *Hystrix*, 26(2), pp. 69–75. doi: 10.4404/hystrix-26.2-11166.

Shuttleworth, C. M., Kenward, R. E. and Jackson, N. (2008) 'Re-introduction of the red squirrel into Newborough forest on the island of Anglesey', in Soorae, P. S. (ed.) *Global re-introduction perspectives: re-introduction case studies from around the globe.* Abu Dhabi: IUCN/SSC Re-introduction Specialist Group, pp. 163–166. Available at: https://portals.iucn.org/library/sites/library/files/documents/2008-028.pdf.

Shuttleworth, Craig M, Lurz, P. W. W. and Halliwell, E. C. (2015) *Shared Experience of Red Squirrel Conservation Practice*. Edited by C. M. Shuttleworth, P. W. W. W. Lurz, and E. C. Halliwell. European Squirrel Initiative. Available at: http://www.redsquirrels.info/wp-content/uploads/2015/09/Red-Squirrel-Conservation-eBook.pdf.

Sikes, R. S. and Gannon, W. L. (2011) 'Guidelines of the American Society of Mammalogists for the use of wild mammals in research', *Journal of Mammalogy*, 92(1), pp. 235–253. doi: 10.1644/10-MAMM-F-355.1.

Silva, C. A. M. *et al.* (2013) 'Interaction of Mycobacterium leprae with human airway epithelial cells: Adherence, entry, survival, and identification of potential adhesins by surface

proteome analysis', *Infection and Immunity*, 81(7), pp. 2645–2659. doi: 10.1128/IAI.00147-13.

Simpson, V. *et al.* (2015) 'Leprosy in red squirrels on the Isle of Wight and Brownsea Island', *Veterinary Record*, 177(8), pp. 206–207. doi: 10.1136/vr.h4491.

Simpson, V. R. *et al.* (2013) 'Causes of mortality and pathological lesions observed post-mortem in red squirrels (Sciurus vulgaris) in Great Britain.', *BMC veterinary research*, 9(1), p. 229. doi: 10.1186/1746-6148-9-229.

Singh, N. and Perfect, J. R. (2007) 'Immune Reconstitution Syndrome and Exacerbation of Infections after Pregnancy', *Clinical Infectious Diseases*, 45, pp. 1192–9. doi: 10.1086/522182.

Singh, P. *et al.* (2015) 'Insight into the evolution and origin of leprosy bacilli from the genome sequence of Mycobacterium lepromatosis.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 112(14), pp. 4459–64. doi: 10.1073/pnas.1421504112.

Singh, P. K., Singh, A. V. and Chauhan, D. S. (2013) 'Current understanding on micro RNAs and its regulation in response to Mycobacterial infections', *Journal of Biomedical Science*, 20(1), pp. 1–9. doi: 10.1186/1423-0127-20-14.

Smith, A. D. H. *et al.* (2010) 'The validity of wireless iButtons® and thermistors for human skin temperature measurement', *Physiological Measurement*, 31(1), pp. 95–114. doi: 10.1088/0967-3334/31/1/007.

Smith, K. F., Acevedo-Whitehouse, K. and Pedersen, A. B. (2009) 'The role of infectious diseases in biological conservation', *Animal Conservation*, 12(1), pp. 1–12. doi: 10.1111/j.1469-1795.2008.00228.x.

Smith, S. H. *et al.* (2017) 'Atypical Histiocytosis in Red Squirrels (Sciurus vulgaris)', *Journal of Comparative Pathology*. Elsevier Ltd, 156(4), pp. 446–450. doi: 10.1016/j.jcpa.2017.02.002.

Smith, W. C. *et al.* (2015) 'The Missing Millions: A Threat to the Elimination of Leprosy', *PLoS Neglected Tropical Diseases*, 9(4), pp. 2–5. doi: 10.1371/journal.pntd.0003658.

Soares, C. T. *et al.* (2017) 'Differential expression of microRNAs in leprosy skin lesions', *Frontiers in Immunology*, 8(AUG), pp. 1–10. doi: 10.3389/fimmu.2017.01035.

Sousa Lima, M. I. *et al.* (2019) 'Molecular and Biotechnological Approaches in the Diagnosis of Leprosy', in Ribon, W. (ed.) *Hansen's Disease - The Forgotten and Neglected Disease*. IntechOpen, pp. 63–80. doi: http://dx.doi.org/10.5772/intechopen.75506.

de Souza, M. M. *et al.* (2014) 'Utility of recombinant proteins LID-1 and PADL in screening for Mycobacterium leprae infection and leprosy', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 108(June), pp. 495–501. doi: 10.1093/trstmh/tru093.

Spencer, J. S. and Brennan, P. J. (2011) 'The role of Mycobacterium leprae phenolic glycolipid I (PGL-I) in serodiagnosis and in the pathogenesis of leprosy.', *Leprosy review*, 82(4), pp. 344–57.

Stallknecht, D. E. *et al.* (1987) 'Surveillance for naturally acquired leprosy in a nine-banded armadillo population.', *Journal of wildlife diseases*, 23(2), pp. 308–310. doi: 10.7589/0090-3558-23.2.308.

Stephen, C. (2014) ‘Toward a Modernized Definition of Wildlife Health’, *Journal of Wildlife Diseases*, 50(3), pp. 427–430. doi: 10.7589/2013-11-305.

Storrs, E. E. *et al.* (1974) ‘Leprosy in the Armadillo: New Model for Biomedical Research.’, *Science*, 183(4127), pp. 851–852. doi: 10.1126/science.183.4127.851.

Storrs, E. E. (1978) ‘Animal model of human disease: Lepromatous leprosy’, *American Journal of Pathology*, 92(3), pp. 813–816.

Suarez-Garcia, I. *et al.* (2017) ‘The decline of autochthonous leprosy in the Valencia Region of Spain : patterns and’, *Leprosy Review*, (88), pp. 162–173.

Suzuki, K. *et al.* (2010) ‘Infection during infancy and long incubation period of leprosy suggested in a case of a chimpanzee used for medical research’, *Journal of Clinical Microbiology*, 48(9), pp. 3432–3434. doi: 10.1128/JCM.00017-10.

Swift, B. M. C., Convery, T. W. and Rees, C. E. D. (2016) ‘Evidence of Mycobacterium tuberculosis complex bacteraemia in intradermal skin test positive cattle detected using phage-RPA’, *Virulence*. Taylor & Francis, 7(7), pp. 779–788. doi: 10.1080/21505594.2016.1191729.

Taher, T. E. *et al.* (2017) ‘Intracellular B Lymphocyte Signalling and the Regulation of Humoral Immunity and Autoimmunity’, *Clinical Reviews in Allergy and Immunology*. Clinical Reviews in Allergy & Immunology, 53(2), pp. 237–264. doi: 10.1007/s12016-017-8609-4.

Talhari, C., Talhari, S. and Penna, G. O. (2015) ‘Clinical aspects of leprosy’, *Clinics in Dermatology*. Elsevier Inc., 33(1), pp. 26–37. doi: 10.1016/j.clindermatol.2014.07.002.

Taulman, J. F. and Robbins, L. W. (2014) ‘Range expansion and distributional limits of the nine-banded armadillo in the United States: An update of Taulman & Robbins (1996)’, *Journal of Biogeography*, 41(8), pp. 1626–1630. doi: 10.1111/jbi.12319.

The Scottish Squirrel Group (2015) ‘Scottish Strategy for Red Squirrel Conservation’, (June).

Tio-Coma, M. *et al.* (2019) ‘Detection of Mycobacterium leprae DNA in soil; Multiple needles in the haystack?’, *Scientific Reports*, (9). doi: https://doi.org/10.1038/s41598-019-39746-6.

Tompkins, D. M., White, A. and Boots, M. (2003) ‘Ecological replacement of native red squirrels by invasive greys driven by disease’, *Ecology Letters*, (6), pp. 186–196.

Truman, R. (2005) ‘Leprosy in Wild Armadillos.’, *Leprosy Review*, 76(3), pp. 198–208.

Truman, R. (2008) ‘Armadillos as a source of infection for leprosy’, *South Med J*, 101(6), pp. 581–582. doi: 10.1097/SMJ.0b013e318172dd6c.

Truman, R. and Fine, P. E. M. (2010) ‘“Environmental” sources of Mycobacterium leprae: issues and evidence.’, *Leprosy review*, 81(2), pp. 89–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20825112.

Truman, R. W. (1985) *Host Responses to the Phenolic-Glycolipid-1 Antigen of Mycobacterium Leprae*. Lousiana State University.

Truman, R. W. *et al.* (1986) ‘Evaluation of the origin of Mycobacterium leprae infections in the wild armadillo, Dasypus novemcinctus’, *American Journal of Tropical Medicine and Hygiene*, 35(3), pp. 588–593. doi: 10.4269/ajtmh.1986.35.588.

Truman, R. W. *et al.* (1991) ‘Seasonal and spatial trends in the detectability of leprosy in wild

armadillos', *Epidemiol Infect*, 106(3), pp. 549–560. doi: 10.1017/S0950268800067613.

Truman, R. W. *et al.* (2011) 'Probable Zoonotic Leprosy in the Southern United States', *The New England Journal of Medicine*, 364(17), pp. 1626–1633.

Truman, R. W. *et al.* (2014) 'The armadillo as a model for peripheral neuropathy in leprosy', *ILAR Journal*, 54(3), pp. 304–314. doi: 10.1093/ilar/ilt050.

Truman, R. W. and Krahenbuhl, J. L. (2001) 'Viable M. leprae as a Research Reagent', *International Journal of Leprosy and Other Mycobacterial Diseases*, 69(1).

Turankar, R. P. *et al.* (2012) 'Dynamics of Mycobacterium leprae transmission in environmental context: Deciphering the role of environment as a potential reservoir', *Infection, Genetics and Evolution*. Elsevier B.V., 12(1), pp. 121–126. doi: 10.1016/j.meegid.2011.10.023.

Turankar, R. P. *et al.* (2016) 'Presence of viable Mycobacterium leprae in environmental specimens around houses of leprosy patients', *Indian J Med Microbiol.*, 34(3), pp. 315–321. doi: 10.4103/0255-0857.188322.

Turankar, R. P. *et al.* (2019) 'Survival of Mycobacterium leprae and association with Acanthamoeba from environmental samples in the inhabitant areas of active leprosy cases: A cross sectional study from endemic pockets of Purulia, West Bengal', *Infection, Genetics and Evolution*. Elsevier, 72(January), pp. 199–204. doi: 10.1016/j.meegid.2019.01.014.

UCDavis (2019) *PREDICT Project | School of Veterinary Medicine*. Available at: https://ohi.vetmed.ucdavis.edu/programs-projects/predict-project (Accessed: 16 August 2019).

Valois, E. M. de S., Campos, F. M. C. and Ignotti, E. (2015) 'Prevalence of Mycobacterium leprae in the environment: A review', *African Journal of Microbiology Research*, 9(40), pp. 2103–2110. doi: 10.5897/AJMR2015.7440.

Valverde, C. R. *et al.* (1998) 'Spontaneous leprosy in a wild-caught cynomolgus macaque.', *International Journal of Leprosy and Other Mycobacterial Diseases*, 66(2), pp. 140–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9728446.

Velarde-Félix, J. S., Alvarado-Villa, G. and Vera-Cabrera, L. (2016) '"Lucio's phenomenon" associated with mycobacterium lepromatosis', *American Journal of Tropical Medicine and Hygiene*, 94(3), pp. 483–484. doi: 10.4269/ajtmh.15-0439.

Vera-Cabrera, L. *et al.* (2015) 'Mycobacterium lepromatosis infections in Nuevo Leon, Mexico', *Journal of Clinical Microbiology*, 53(6), pp. 1945–1946. doi: 10.1128/JCM.03667-14.

Viana, M. *et al.* (2014) 'Assembling evidence for identifying reservoirs of infection', *Trends in Ecology and Evolution*, 29(5), pp. 270–279. doi: 10.1016/j.tree.2014.03.002.

Villada, G. *et al.* (2016) 'Autochthonous borderline tuberculoid leprosy in a man from Florida.', *Leprosy review*, 87(1), pp. 101–103.

Virk, A. *et al.* (2017) 'Mycobacterium lepromatosis: Lepromatous leprosy in US Citizen Who Traveled to Disease-Endemic Areas.', *Emerging Infectious Diseases*, 23(11), pp. 1864–1866. doi: 10.1590/0074-02760170117.

Virmond, M., Grzybowski, A. and Virmond, L. (2015) 'Leprosy: A glossary', *Clinics in Dermatology*. Elsevier B.V., 33(1), pp. 8–18. doi: 10.1016/j.clindermatol.2014.07.006.

Walsh, G. P. *et al.* (2012) ‘Limited susceptibility of cynomolgus monkeys (Macaca fascicularis) to leprosy after experimental administration of Mycobacterium leprae’, *American Journal of Tropical Medicine and Hygiene*, 87(2), pp. 327–336. doi: 10.4269/ajtmh.2012.11-0780.

Walsh, G. P., Meyers, W. M. and Binford, C. H. (1986) ‘Naturally Acquired Leprosy in the Nine- Banded Armadillo: A Decade of Experience 1975 - 1985’, *Journal of Leukocyte Biology*, 40(5), pp. 645–656.

Wauters, L. A. *et al.* (2017) ‘New endemic mammal species for europe: Sciurus meridionalis (Rodentia, Sciuridae)’, *Hystrix*, 28(1), pp. 1–8. doi: 10.4404/hystrix-28.1-12015.

Webb, R. C. *et al.* (2013) ‘Ultrathin conformal devices for precise and continuous thermal characterization of human skin’, *Nature Materials*. Nature Publishing Group, 12(10), pp. 938–944. doi: 10.1038/nmat3755.

Wen, Y. *et al.* (2013) ‘Whole-blood nested-PCR amplification of M. leprae-specific DNA for early diagnosis of leprosy’, *American Journal of Tropical Medicine and Hygiene*, 88(5), pp. 918–922. doi: 10.4269/ajtmh.11-0253.

Wernike, K. *et al.* (2018) ‘Widespread occurrence of squirrel adenovirus 1 in red and grey squirrels in Scotland detected by a novel real-time PCR assay’, *Virus Research*. Elsevier, 257(August), pp. 113–118. doi: 10.1016/j.virusres.2018.09.007.

Wheat, W. H. *et al.* (2014) ‘Long-term Survival and Virulence of Mycobacterium leprae in Amoebal Cysts’, 8(12). doi: 10.1371/journal.pntd.0003405.

White, A. *et al.* (2016) ‘Modelling disease spread in real landscapes: Squirrelpox spread in Southern Scotland as a case study.’, *Hystrix, the Italian Journal of Mammalogy*, 27(1).

White, C. and Franco-Paredes, C. (2015) ‘Leprosy in the 21st century’, *Clinical Microbiology Reviews*, 28(1), pp. 80–94. doi: 10.1128/CMR.00079-13.

WHO (2016a) *Global Leprosy Strategy 2016-2020 - Accelerating towards a leprosy-free world*. New Delhi.

WHO (2016b) ‘Global leprosy update, 2015: time for action, accountability and inclusion’, *World Health Organisation Weekly epidemiological record*, 91(35), pp. 405–420. doi: 10.1186/1750-9378-2-15.Voir.

WHO (2017) *Guidelines for the Diagnosis, Treatment and Prevention of Leprosy*.

WHO (2018a) ‘Global leprosy update, 2017: reducing the disease burden due to leprosy’, *Weekly Epidemiological Record*, 93(35), pp. 445–456. doi: 10.1177/1045389X06056341.

WHO (2018b) ‘WHO | Life expectancy’, *WHO*. World Health Organization. Available at: https://www.who.int/gho/mortality_burden_disease/life_tables/situation_trends/en/ (Accessed: 18 June 2019).

Wibbelt, G. *et al.* (2017) ‘Berlin squirrelpox virus, a new poxvirus in red squirrels, Berlin, Germany’, *Emerging Infectious Diseases*, 23(10), pp. 1726–1729. doi: 10.3201/eid2310.171008.

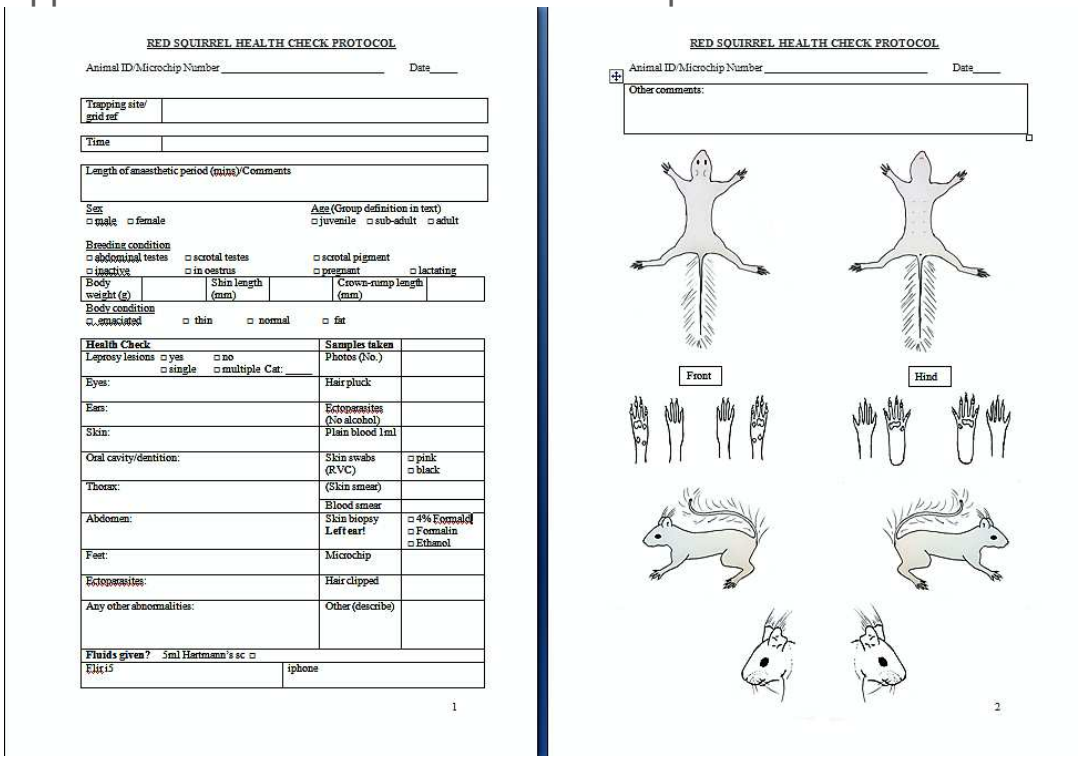
Williams, A. J. and Loughry, W. J. (2012) ‘Temporal Aspects of Leprosy Infection in a Wild Population of Nine-Banded Armadillos’, *Southeastern Naturalist*, 11(2), pp. 173–182. doi: 10.1656/058.011.0202.

Woodford, M. H. (2000) *Quarantine and health screening protocols for wildlife prior to translocation and release into the wild*, *Published jointly by the IUCN Species Survival Commission's Veterinary Specialist Group, Gland, Switzerland, the Office International des Epizooties (OIE), Paris, France, Care for the Wild, U.K., and the European Association of Zoo and Wildlife Veterinari*. Available at: http://digitalcommons.unl.edu/zoonoticspubhttp://digitalcommons.unl.edu/zoonoticspub/32.

Zhu, Y. and Sabuncu, M. R. (2018) 'A Probabilistic Disease Progression Model for Predicting Future Clinical Outcome'. Available at: http://arxiv.org/abs/1803.05011.

Appendices

Appendix I: General health assessment protocol



RED SQUIRREL HEALTH CHECK PROTOCOL

Animal ID/Microchip Number _____ Date____

Trapping site/ grid ref	
Time	
Length of anaesthetic period (mins)/Comments	

Sex
□ male □ female

Age (Group definition in text)
□ juvenile □ sub-adult □ adult

Breeding condition
□ abdominal testes □ scrotal testes □ scrotal pigment
□ inactive □ in oestrus □ pregnant □ lactating

Body weight (g)		Shin length (mm)		Crown-rump length (mm)	

Body condition
□ emaciated □ thin □ normal □ fat

Health Check		Samples taken	
Leprosy lesions □ yes □ no □ single □ multiple Cat: ____		Photos (No.)	
Eyes:		Hair pluck	
Ears:		Ectoparasites (No alcohol)	
Skin:		Plain blood 1ml	
Oral cavity/dentition:		Skin swabs (RVC)	□ pink □ black
Thorax:		(Skin smear)	
		Blood smear	
Abdomen:		Skin biopsy Left ear!	□ 4% Formalin □ Formalin □ Ethanol
Feet:		Microchip	
Ectoparasites:		Hair clipped	
Any other abnormalities:		Other (describe)	

Fluids given? 5ml Hartmann's sc □

Flir i5	iphone

1

RED SQUIRREL HEALTH CHECK PROTOCOL

Animal ID/Microchip Number _____ Date____

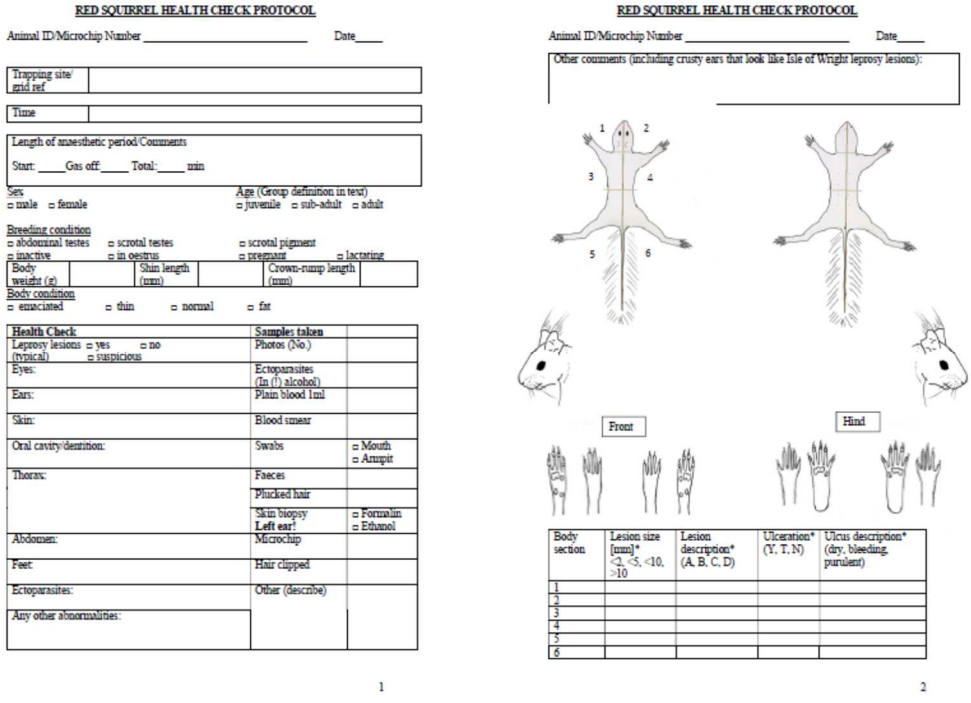
Other comments:

Front

Hind

2

FIGURE 86: ORIGINAL HEALTH CHECK PROTOCOL



RED SQUIRREL HEALTH CHECK PROTOCOL

Animal ID/Microchip Number _____ Date____

Trapping site/ grid ref	
Time	
Length of anaesthetic period/Comments Start: ____Gas off____ Total:____ min	

Sex
□ male □ female

Age (Group definition in text)
□ juvenile □ sub-adult □ adult

Breeding condition
□ abdominal testes □ scrotal testes □ scrotal pigment
□ inactive □ in oestrus □ pregnant □ lactating

Body weight (g)		Shin length (mm)		Crown-rump length (mm)	

Body condition
□ emaciated □ thin □ normal □ fat

Health Check		Samples taken	
Leprosy lesions □ yes □ no (typical) □ suspicious		Photos (No.)	
Eyes:		Ectoparasites (In (!) alcohol)	
Ears:		Plain blood 1ml	
Skin:		Blood smear	
Oral cavity/dentition:		Swabs	□ Mouth □ Armpit
Thorax:		Faeces	
		Plucked hair	
		Skin biopsy Left ear!	□ Formalin □ Ethanol
Abdomen:		Microchip	
Feet:		Hair clipped	
Ectoparasites:		Other (describe)	
Any other abnormalities:			

1

RED SQUIRREL HEALTH CHECK PROTOCOL

Animal ID/Microchip Number _____ Date____

Other comments (including crusty ears that look like Isle of Wright leprosy lesions):

1 2 3 4 5 6

Front

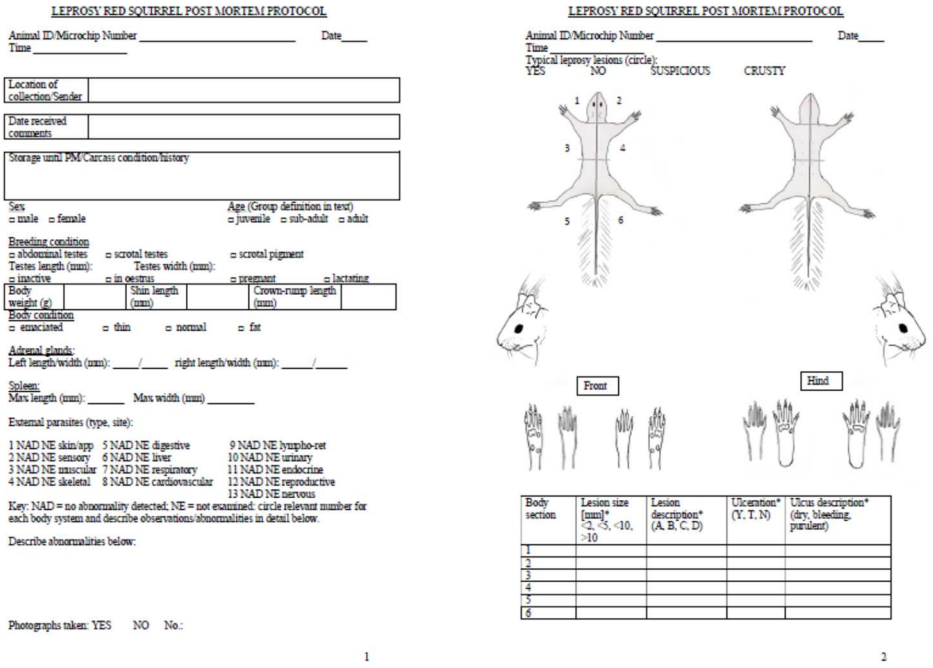
Hind

Body section	Lesion size [mm]* <2, <5, <10, >10	Lesion description* (A, B, C, D)	Ulceration* (Y, T, N)	Ulcus description* (dry, bleeding, purulent)
1				
2				
3				
4				
5				
6				

2

FIGURE 87: FINAL HEALTH CHECK PROTOCOL

Appendix II: Post mortem protocol



LEPROSY RED SQUIRREL POST MORTEM PROTOCOL

Animal ID/Microchip Number _____ Date____
Time _____

Location of collection/Sender	
Date received comments	
Storage until PM/Carcass condition/history	

Sex
□ male □ female

Age (Group definition in text)
□ juvenile □ sub-adult □ adult

Breeding condition
□ abdoominal testes □ scrotal testes □ scrotal pigment
Testes length (mm): Testes width (mm):
□ inactive □ in oestrus □ pregnant □ lactating

Body weight (g)		Shin length (mm)		Crown-rump length (mm)	

Body condition
□ emaciated □ thin □ normal □ fat

Adrenal glands:
Left length/width (mm): ____/____ right length/width (mm): ____/____

Spleen:
Max length (mm): _____ Max width (mm) _____

External parasites (type, site):

1 NAD NE skin/app 5 NAD NE digestive 9 NAD NE lympho-ret
2 NAD NE sensory 6 NAD NE liver 10 NAD NE urinary
3 NAD NE muscular 7 NAD NE respiratory 11 NAD NE endocrine
4 NAD NE skeletal 8 NAD NE cardiovascular 12 NAD NE reproductive
13 NAD NE nervous

Key: NAD = no abnormality detected; NE = not examined: circle relevant number for each body system and describe observations/abnormalities in detail below.

Describe abnormalities below:

Photographs taken: YES NO No.:

1

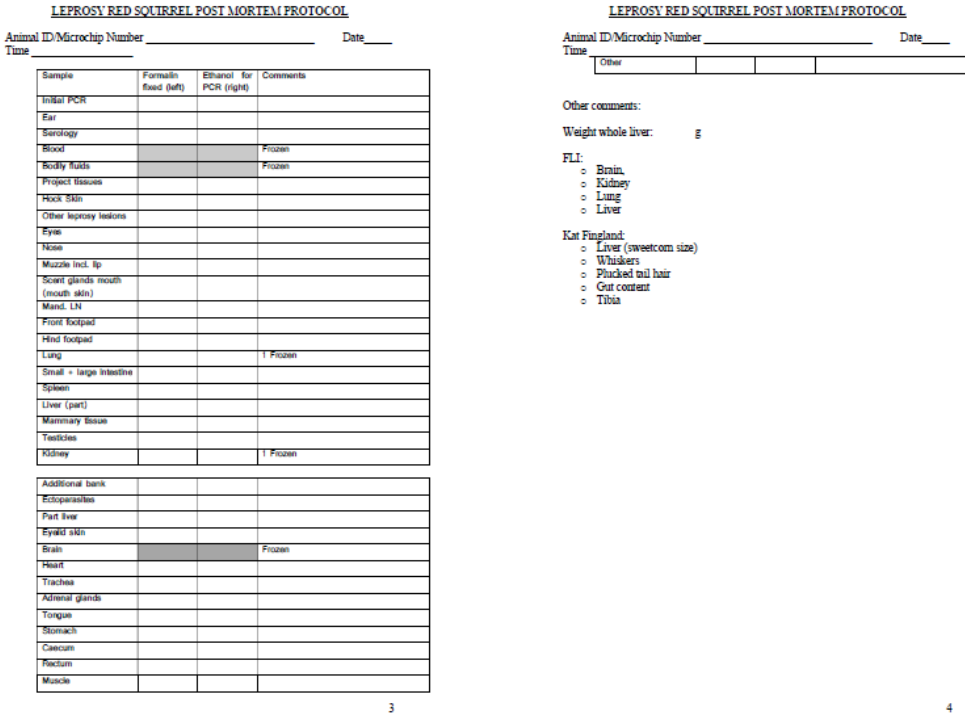
LEPROSY RED SQUIRREL POST MORTEM PROTOCOL

Animal ID/Microchip Number _____ Date____
Time
Typical leprosy lesions (circle):
YES NO SUSPICIOUS CRUSTY

Body section	Lesion size [mm]* <2, <5, <10, >10	Lesion description* (A, B, C, D)	Ulceration* (Y, T, N)	Ulcus description* (dry, bleeding, purulent)
1				
2				
3				
4				
5				
6				

2

FIGURE 88: FRONT PAGE OF THE SQUIRREL POST MORTEM PROTOCOL USED IN THIS PROJECT



LEPROSY RED SQUIRREL POST MORTEM PROTOCOL

Animal ID/Microchip Number _____ Date____
Time _____

Sample	Formalin fixed (left)	Ethanol for PCR (right)	Comments
Initial PCR			
Ear			
Serology			
Blood			Frozen
Bodily fluids			Frozen
Project tissues			
Hock Skin			
Other leprosy lesions			
Eyes			
Nose			
Muzzle incl. lip			
Scent glands mouth (mouth skin)			
Mand. LN			
Front footpad			
Hind footpad			
Lung			1 Frozen
Small + large intestine			
Spleen			
Liver (part)			
Mammary tissue			
Testicles			
Kidney			1 Frozen

Additional bank			
Ectoparasites			
Part liver			
Eyelid skin			
Brain			Frozen
Heart			
Trachea			
Adrenal glands			
Tongue			
Stomach			
Caecum			
Rectum			
Muscle			

3

LEPROSY RED SQUIRREL POST MORTEM PROTOCOL

Animal ID/Microchip Number _____ Date____
Time _____

Other			

Other comments:

Weight whole liver: g

FLI:
- Brain
- Kidney
- Lung
- Liver

Kat Fingland:
- Liver (sweetcorn size)
- Whiskers
- Plucked tail hair
- Gut content
- Tibia

4

FIGURE 89: SECOND PAGE OF POST MORTEM PROTOCOL

Appendix III: Post mortem findings

CR= crown-rump length, NAD= No abnormalities detected, NA= Not assessed, no measurements or BCS given when carcass to destroyed to determine

Animal	State of carcass	General information	Skin	Other observations	*M. leprae* DNA
BIC001_16	Frozen immediately post euthanasia	Adult, female, late lactation, normal BCS, 345g, 185 mm CR, 71 mm shin	Leprosy lesion (score 39, cat.4), leprosy lesion on left hock not quite usual appearance, some in skin, but also swelling of the joint capsule	Mandibular lymph nodes enlarged, lung not fully ventilated, right lung very dark, not floating, intranasal mass (3mm diameter) in right conchae, left adrenal slight mustard colour, maybe enlarged, three small white spots, intestines quite red, gas in stomach and intestines, some food, uterus well retracted despite still lactating, very deep in pelvis, left kidney basin maybe slightly widened, liver 15.1g	Present
BIC002_16	Frozen shortly after death	Adult, male, scrotal testes, emaciated, 300g, 164 mm CR, 63.4mm shin	Leprosy lesion (score 38, cat.4), Leprosy lesions on both ears, the nose, both hocks and on scrotum and preputium	Testes actually inguinal, displaced by scrotal and preputial mass; buccal abscess left head side reaching all the way to the mandibula, skin can be pulled off, oral cavity/teeth NAD, mandibular lymph node enlarged; lungs and trachea filled with red foamy fluid, pericardium filled with red fluid; liver very firm, tweezer imprints stay for a long time, intestines reddened some sections of small and large intestine and rectum leathery with white specs on them; white imprint of intestines on stomach; white matter in fur next to scrotum, swelling of lateral toe right front foot	Present
BIC003_16	Frozen shortly after death	Adult, female, pregnant, emaciated, 295 g, 174 mm CR, 66 mm shin	Leprosy lesion (score 23, cat.4); Leprosy lesions left ear and right hock mild, right ear and left hock severe, very thick coat and long ear tufts for June	Skull base fractured from airgun shot; stomach well filled, intestines only little filling, gastro-intestinal tract throughout, reddening; liver slightly off colour/with a shade of yellow; caudal lungs NAD, cranial lungs filled with red liquid; left kidney slightly pale and larger than right	Present

BIC004_16	Frozen not very long after death, skin slightly dried out, beginning autolysis of internal organs	Subadult, female, inactive, emaciated, 125g, 132mm CR, 56.1mm shin	No leprosy lesions	Lung filled with red coloured foam and marmorised appearance (dark streaks), lung itself pale yellowish, floats, small amount of red, clear liquid in thorax and pericard; some mediastinal lymph nodes of red colour, faecal matter in intestines and formed faeces in fur	Absent
BIC005_16	Frozen after some decay	Adult, male, pigmented scrotum, normal BCS, 300 g rest-weight, 180 mm CR, 67.9mm shin	No leprosy lesions	Eyes autolytic, maggots under skin, in thorax and liver; no right kidney found; stomach and small intestine mildly filled with food, caecum, large intestine and rectum massively filled with light brown crumbly food paste, no obvious obstruction, rectal wall reddened	Present
BIC006_16	Frozen shortly after death	Adult, female, inactive, fat, 430g (wet), 175mm CR, 62.4mm shin	No leprosy lesions	Liver appears rather large (18.8g); lung and trachea filled with foamy red liquid; red fluid in pericardium and thorax; stomach large, filled mainly with fluid; animal has bred before but is currently reproductively inactive	Present
BIC007_16	Frozen shortly after death	Adult, male, abdominal testes, emaciated, 225g, 169mm CR, 66.3mm shin	Leprosy lesion (score 25, cat.4), cranial rim and inside of left ear two small lesions, right hook looks closed lesion, left hook larger, ulcerated lesion	Right front footpad appears swollen; mesenteric vessels filled with blood, dark red areas in neck; lung pink-red marmorised, floats, but red liquid seeping of cut surfaces, liver 10g	Present
BIC008_16	Partly eaten by bird of prey	Adult, female, inactive, normal BCS, 175g (rest weight), 66.6mm shin	No leprosy lesions	Most of head, upper body, and internal organs eaten by bird of prey; only rectum, kidneys, bladder and uterus left for assessment, NAD	Absent
BIC009_16	Frozen shortly after death	Adult, male, abdominal testes, thin, 240g, 165mm CR, 66.5mm shin	No leprosy lesions; left hind footpad appears scaly and there are sores between the toes	Large number of fleas still present on carcass; intestines from stomach to rectum appear reddened; stomach wall very thick, stomach filled with black gravely substance; liver 9.3g; lungs filled with red liquid	Present

BIC010_16	Frozen immediately post euthanasia (not project related)	Adult, female, in oestrus, normal BCS, 365g, 178mm CR, 71.1mm shin	Leprosy lesion (score 4, cat.1), lesion on right hock	Found with hind legs paralysed; euthanised by veterinarian in hospital; lung, hearth and liver cannot be assessed due to severe crystallisation in these organs following euthanasia; lumbosacral region of spine appears overly mobile, but not crepitation and no bleeding into surrounding muscle; free clear, dark red liquid in thorax and abdomen; fluid in bladder mildly reddened	Present
BIC011_16	Mummified	Adult, 170mm CR, 62.8mm shin	NA	NA	Absent
BIC012_17	Mummified	Adult, 60.6mm shin	NA	NA	NA
BIC013_17	Mummified	Adult, 63.5mm shin	NA	NA	NA
BIC014_17	Frozen immediately post euthanasia	Adult, female, in oestrus, thin, 340g, 181mm CR, 69.6mm shin	Leprosy lesion (score 51, cat.4); large ulcerated lesions on ears and hocks, non-ulcerated lesion on nose	Peritoneum and omentum quite red, probably freezing artefact; stomach well filled, but intestines not; very strong blood supply to uterus; liver 13.3g; right caudal lung liver like and only partially floating, mild crystal formation in lung following euthanasia	Present
BIC015_17	Frozen ca. 24h after death	Juvenile, male abdominal testes, emaciated, 110g, 120mm CR, 51.4mm shin	No leprosy lesions	Blood coagula in atria, very thin atrial walls; some red liquid around nose; no obvious bruises or other signs of blunt trauma expected after a fall; Lungs pink and soft elastic	Absent
BIC016_17	Frozen immediately post euthanasia	Adult, female, inactive, emaciated, 310g, 162mm CR, 64.1mm shin	Leprosy lesion (score 15, cat.4), ulcerated lesion on left ear, closed lesions on both hocks	Both adrenal glands relatively large (10x4 and 10x7 mm), left gland pear shaped and pale; muscular swelling around right elbow, muscle appears pale, joint swelling left carpus and right tarsus, capsule thickened, minimal increased liquid filling in right tarsus; uterus very intensely vascularised; crystal formation following euthanasia in liver (16g), heart and lung	Present
BIC017_17	Frozen shortly after death	Juvenile, male, abdominal testes, thin, 105g,	No leprosy lesions	Small amount of red liquid in thorax, caudal lung lobes dark red, only cranial tips of cranial lobes	Absent

		135mm CR, 55.1mm shin		still pink colour; no bruises or broken bones; spleen very pale	
BIC018_18	Advanced autolysis, skull and thoracic organs removed	Adult, female, inactive, BCS cannot be assessed, 310g (rest weight), 68.7mm shin	No leprosy lesions	Skull and thoracic organs removed post mortem, significant manipulation of carcass assumed; right caudal body wall ruptured; some intestinal invagination; abdominal organs advanced autolysis; liver 7.4g.	Present
BIC019_18	Frozen after some exposure to elements, some maggots	Adult, female, inactive, emaciated, 350g, 170mm CR, 67.9mm shin	No leprosy lesions, small areas of hair loss on left muzzle and hocks	Lactated before, currently reproductively inactive; some free red liquid in thorax (~3ml) and abdomen (2ml); gas build up in stomach and intestines, rectum widened; stomach and gut contents very dark (almost black) and liquid, some soiling on hind legs, probably diarrhoea ante mortem; right atrium enlarged, cardiac muscle appears contracted in some areas (scars?); some lung sections darkened, brown fluid can be pressed of cut surface	Present
BIC020_18	Frozen shortly after death	Juvenile, male, abdominal testes, emaciated, 90g, 103mm CR, 49mm shin	No leprosy lesions	Digestive tract empty, only mild gas build up; very thin muscle cover, no fat cover internally or externally; lungs darkened in several sections; few fleas visible	Absent
BIC021_18	Frozen after some exposure to elements, some maggots	Juvenile, male, abdominal testes, thin, 135g, 133mm CR, 60.5mm shin	No leprosy lesions	More than 15 fleas, some engorged ticks; no internal fat cover, oral cavity completely filled with freshly hatched maggots, some in nose as well; soiling around anus and hind legs, very liquid intestinal content, in sections intestines reddened and content red liquid, intestinal wall in some segments thickened, particularly thick caecum; mandibular lymph nodes enlarged; some free red fluid (<2ml) in body cavity	Present
ARC001_17	Frozen shortly after death, roadkill	Adult, male, abdominal testes, normal BCS,	No leprosy lesions	Multiple fractures including skull and left tibia and fibula; body wall of left caudal abdomen ruptured, trachea ruptured, lungs collapsed; "fat eyes" floating on red fluid in body cavity; little food in	Absent

		355g, ca. 180mm CR, 67.3mm shin		stomach and intestines; good fat cover of the body and organs	
ARC002_17	Frozen shortly after death, predated?	Adult, female, inactive, normal BCS, 315g, 171mm CR, 68.6mm shin	No leprosy lesions	Wound under left elbow: ca. 3mm wound penetrating into thorax, lungs collapsed, ~3ml red fluid in thorax; mucosa pale, no broken bones, intestines barely filled, but stomach well filled with nut and blackberry; liver pale, 9.3g	Absent
ARC003_17	Frozen after onset of initial autolysis, footpads dried, roadkill	Adult, female, inactive, 320g, 67.4mm shin	No leprosy lesions	Open fracture right elbow; skull crushed and deformed; otherwise NAD	Absent
ARC004_17	Frozen after being in road for some time, run over several times, roadkill	Adult, sex and BCS NA, 310g, 71mm shin	No leprosy lesions	Carcass too damaged for full post mortem assessment	Absent
ARC005_17	Frozen after onset of initial autolysis, roadkill	Adult, female, inactive, 340g, 165mm CR, 71.4mm shin	No leprosy lesions	Multiple fractures to the pelvis; left femur fractured, 3-5 digit nails and last phalanx scrapped off on right hind foot, liver 7.8g, some lung sections very dark but still floating, digestive tract quite empty	Absent
ARC006_17	Frozen after being on road for some time, some scavenging, roadkill	Adult, female, inactive, normal BCS, 340g, 188mm CR, 71.9mm shin	No leprosy lesions	Crushed skull, eyes destroyed; tongue, intestines, and left kidney missing; pelvis exposed; liver 11.2g	Absent
ARC007_17	Frozen shortly after death, roadkill	Adult, female, inactive, normal BCS, 365g, 173mm CR, 67.4mm shin	No leprosy lesions	Crushed skull, brain mass pressed out of skull in between skin and cartilage of the left ear; liver 10.7g NAD; lung may have had some changes ante mortem, very difficult to tell from changes (darkening, marmorisation) than could have occurred post mortem.	Absent
ARC008_17	Frozen shortly after death, roadkill	Adult, male, abdominal testes, thin, 365g, 173mm CR, 67.4mm shin	No leprosy lesions	Larval and nymph ticks present, but poorly engorged; left shin and skull fractured; caudal right abdominal wall ruptured, intestines under skin; liver 7.8g, ruptured, quite pale; pericardium ruptured; lungs covered with coagulated red mass;	Absent

				lungs generally in good condition and very pink only small sections of caudal lobes darkened	
ARC009_17	Frozen min. 24 h after death, autolysis of internal organs, maggots present, roadkill	Adult, female, inactive, normal BCS, 340g, 168mm CR, 70.4mm shin	No leprosy lesions	Spine fractured and left abdominal wall ruptured, intestines along leg and some outside the body; maggots focussed on head/oral cavity; skin and superficial muscle layers dried out; large amount of dark fluid in thorax; lungs very dark; spleen cannot be identified	Absent
ARC010_17	Frozen after onset of initial autolysis, roadkill	Subadult, male, abdominal testes, normal BCS, 290g, 68.9mm shin	No leprosy lesions	Pelvis, left femur and skull fractured; heart ruptured from impact, large amount of coagulated red fluid in thorax; mild changes to the lung, but mostly pink, well inflated; trachea ruptured; liver 6.2g ruptured; stomach and small intestine fairly empty, some faecal matter in caecum and large intestines	Absent
ARC011_17	Frozen shortly after death, roadkill	Adult, female, inactive. 375g, 171mm CR, 71.6mm shin	No leprosy lesions	Pelvis, ribcage, skull, right radius and ulna fractured; diaphragm ruptured, some intestines in thorax, some lung in abdomen; brain destroyed; liver 8g, ruptured in several locations; lung and trachea ruptured; right eye destroyed, atria ruptured, kidneys very dark red (cortex)	Absent
ARC012_17	Frozen after onset of initial autolysis, roadkill	Adult, male, abdominal testes, normal BCS, 360g, 170mm CR, 66.9mm shin	No leprosy lesions	NAD	Absent
ARC013_17	Frozen shortly after death, roadkill	Adult, female, inactive, normal BCS, 355g, 168mm CR, 65.1mm shin	No leprosy lesions	Skull fractured	Absent
ARC014_17	Frozen shortly after death, roadkill	Adult, male, scrotal pigment, normal body condition, 305g,	No leprosy lesions	Very young adult, probably from late litter year before, winter coat; skull and ribcage fractured; right testicle scrotal, left abdominal; intestines mostly empty, only small amount of food in stomach; good fat cover of internal organs; liver	Absent

		163mm CR, 65.8mm shin		8.5 g; trachea ruptured large amounts of red fluid in thorax, some coagulated	
ARC015_17	Frozen shortly after death, roadkill	Adult, female, inactive, thin, 220g, 162mm CR, 68.4mm shin	No leprosy lesions	Very young adult; no internal fat cover at all; ticks and fleas observed, whole carcass appears anaemic; skull fractured, bleeding into skull; very pale; empty stomach, large intestine filled, some content very yellow; kidney's very beige; liver 5.4g pale; lung mostly pink, small section in cranial lobes darkened	Absent
ARC016_17	Frozen shortly after death, roadkill	Adult, male, abdominal testes, thin, 330g, 68.5mm shin	No leprosy lesions	Skull and ribcage fractured; trachea and aorta ripped; heart dislocated into abdominal cavity; little food in stomach and GIT	Present
ARC017_18	Frozen immediately after sudden death	Adult, female, pregnant, thin, 430g, 180mm CR, 70.4mm shin	No leprosy lesions	Despite spine and hip bones being easily palpable, the animal had very good intestinal and sternal fat stores; pregnant (right horn), 4 foetus SSL 2cm, silvery firm elastic tumour to the right of the vulva caudal of last mammary complex(1.1x0.8x0.5 cm); stomach very full with peanut mush, dilated, small amount of food in small intestine, large intestine mostly empty, caecum dilated, content brown liquid, GIT generally quite red in colour, lymph nodes in mesenterium clearly visible; liver 14.8g, caudate lobe appears large, some discoloration around gall bladder; kidneys appear dark in colour, well covered in fat; lungs very dark and firm, dark sections do not float, right heart dilated, wall thin, some coagulated blood in pericardium	Absent
ARC018_18	Frozen immediately post euthanasia	Adult, male, scrotal pigment, normal BCS, 340g, 171mm CR, 71.4mm shin	No leprosy lesions	Observed in garden on squirrel feeder with first one, then both front feet missing. Trapped and taken to Hessilhead wildlife rescue, euthanised as not deemed fit to survive in the wild. Lung and heart cannot be assessed (crystallisation); both stumps dry and clean looking, skin healing slightly more advanced on the right side. Right paw taken	Absent

				of through carpal joint, cut surface on bone very clean, left paw taken off slightly above carpus through radius and ulna, cutting side shows signs of calcification; stomach very full (50g) with nut material, small intestine fairly empty, caecum and large intestine average filling; liver 8g NAD; some abdominal fat, generally good condition for end of winter despite the disability	
ARC019_18	Frozen shortly after death, roadkill	Adult, male, scrotal pigment, thin, 390g, 177mm CR, 69.7mm shin	No leprosy lesions	Skull fractured, healed tail fracture; digestive tract pale, well filled, chewed up nuts in stomach, caecum very well filled with fluid of colour and consistency of dark, thick pea soup, other gut sections mildly filled; tongue tip (1cm) ripped off rest of tongue; liver pale, 10.3g; small amount of clear reddish liquid in thorax; white foam in trachea; some lung sections pink, some very dark red, red fluid can be pressed from dark section, dark sections still floating; right atrium appears enlarged, but normal wall thickness, intra-atrial wall may have had an opening prior to dissection, good internal fat cover	Absent
ARC020_18	Frozen shortly after death, roadkill	Adult, male, scrotal pigment, 345g, 181mm CR, 69.4mm shin	No leprosy lesions	Scrotum opened (no blood in area, possibly opened by scavenger); opening in left body wall, intestines protruding from openings; pelvic fracture; digestive tract well filled, not all parts can still be identified; spleen missing; liver destroyed, partly missing; base of skull fractured; heart destroyed, only part lung remains, nice and pink	Absent
ARC021_18	Frozen sometime after death, maggots on outside of carcass, roadkill	Adult, male, abdominal testes, 335g, 70.1mm shin	No leprosy lesions	Ruptured trachea filled with dark red foam; endoparasites in rectum; right thorax 2 broken ribs and spine broken between 2nd and 3rd thoracic vertebra; liver 7.8g	Absent
ARC022_18	Frozen immediately after sudden death	Adult, female, inactive, thin,	No leprosy lesions	Liver appears enlarged, 19g; some red peritracheal fluid, might be due to cutting into jugular, small amount of red fluid in pericardial	Present

		380g, 179mm CR, 67mm shin		sac; left atrium slightly larger than right; lung very dark in colour only about 15% of tissue of normal appearance and still floating	
ARC023_18	Frozen immediately after sudden death	Adult, male, abdominal testes, normal BCS, 335g, 175mm CR, 72.2mm shin	No leprosy lesions	Some gas and fluid among normal gastrointestinal contents; liver 12g; ca. 1/3 of lung darkened, 1/6 very dark not floating, rest of lung pink-grey and puffy; right heart wall extremely thin, enlarged	Absent
ARC024_18	Frozen sometime after death, skin dried out, no maggots, roadkill	Adult, female, inactive, 350g, 69mm shin	No leprosy lesions, skin abrasion on left elbow,	Fractured pelvis, most ribs broken; body wall ruptured; intestines prolapsed through rectum; stomach and liver ruptured; liver partly in thorax; heart not clearly identifiable; trachea ruptured	Absent
ARC025_18	Frozen shortly after death, roadkill	Adult, male, scrotal pigment, 320g, 177mm CR, 69.4mm shin	No leprosy lesions	Skull, right femur, pelvis and right ribcage fractured; testicles in body cavity; diaphragm ruptured; tongue bitten off; subcutaneous bleeding right thorax; free red fluid in thorax and body cavity; lung and heart NAD	Absent
ARC026_18	Frozen shortly after death, some small maggots in nose, roadkill	Adult, female, inactive, 365g, 75.2mm shin	No leprosy lesions	Multiple fractures; diaphragm ruptured; right heart appears slightly dilated; lung very dark red in some areas; free red fluid in body cavities	Absent
ARC027_18	Frozen shortly after death, roadkill	Adult, female, inactive, normal BCS, 340g, 170mm CR, 71.1mm shin	No leprosy lesions	Liver 6 g; very straight fracture on skull base; otherwise NAD	Absent
ARC028_18	Frozen shortly after death, some scavenging, roadkill	Adult male, abdominal testes, thin, 320g, 72.8mm shin	No leprosy lesions; soft elastic swelling (5x5mm) in left hind footpad, skin intact	Skull and left wrist shatter fractures; abdominal wall ruptured; liver ruptured; diaphragm ruptured	Absent
ARC029_18	Frozen shortly after death, likely run over more than once by car, roadkill	Adult, male, abdominal testes, normal BCS, 350g, 163mm, 70.2mm shin	No leprosy lesions	Tibia and fibula of left hind leg and skull fractured; body wall ruptured, some intestines under skin	Present

Appendix IV: Histological findings

In 11 ear sections some inflammation was observed, in eight it was characterised as chronic-granulomatous, in two just as chronic. In one section autolysis was too severe to characterise the inflammation, in another milder signs of autolysis were present. One was impaired by freezing artefacts (vacuoles). Signs of ulceration were detected in two ear sections. Increased numbers of lymphocytes were seen in all 11 ear sections, macrophage numbers were also increased in all, in eight these were foamy. Epithelioid cells were only seen in one section, as were histiocytes. Where AFB were seen in the ear section, they were mainly intact, only in one fragmentation was noted. Two lesions were limited to an area close to the ear cartilage, five expanded through the dermis, two reached from the cartilage to almost the epidermis and one from the dermis to the epidermis. A clear Unna-band was seen in four sections. In nine sections perineural and perivascular inflammatory infiltrate was observed. In one all normal structure had been destroyed and been replaced by the infiltrate. Periadnexal infiltration was present in three sections in five the adnexe could no longer be clearly identified, in two no periadnexal infiltration occurred. In two non-clinical ears the inflammatory infiltrate was only located close to the cartilage, in two others it was seen in the dermis. In two severe cases is reached from the cartilage to the epidermis, in the other three it was located in the dermis, in the last severe case it reached from the dermis to the epidermis. It is interesting to see that mild/early lesions appear to be located deeper in the tissue.

In eight hock skin sections inflammation was seen, in four instances chronic granulomatous, in two granulomatous and in two too minimal to give it a label. In one section some autolysis was observed. Interestingly the cells within the leprosy lesion appeared less affected than the healthy tissue. One hock skin section showed signs of ulceration. All had increased numbers of lymphocytes, three featured foamy macrophages, four others had increased numbers of macrophages and only in one no increase in these cells was seen. Epithelioid cells were only seen in one section, as were neutrophils, a multinucleated giant cell, and a Langhans cell. Where AFB were seen they were mainly intact, in two sections some AFBs showed signs of fragmentation. The inflammation was limited to the deep dermis in two sections, reaching from here into the dermis in another. It was located in the dermis in four sections and touched the epidermis in one. A clear Unna-band was identified in two sections. Perineural infiltration was seen in four sections, in three the inflammatory infiltrate had largely replaced normal structures. Perivascular infiltration could still be observed in some areas of six sections. Periadnexal infiltration was seen in one section, in three the adnexe could no longer be identified, in four no periadnexal infiltration occurred. In the non-clinical and mild case in which a histological lesion was seen in the hock it was located in the deep dermis or dermis, in the severe cases it was still twice located in the deep dermis, thrice in the dermis and only once reaching the epithel.

In five nose sections chronic-granulomatous inflammation could be identified. In one other it is likely to have been present, but severe autolysis hindered clear classification. Lymphocyte aggregates and foamy macrophages were present in all five analysable sections. A multinucleated giant cell was seen in one section. Intact AFB were present in four nose sections, while they were fragmented in two. In two nose sections the main infiltrate was limited to the area close to the bone/cartilage, in the other three it expanded from here to the olfactory epithel (inner nose). Perineural infiltration was noted in three sections, perivascular in four, and periadnexal infiltration was noted once. All cases in which inflammation in the nasal tissue was seen were classed as severe. It is interesting to note that the inflammation was mainly located in the inner nose, not the outer skin.

In one mandibular lymph node section a granulomatous inflammation could be identified. Two others were suspicious but severe autolysis hampered the analysis. In the best conserved section lymphocytes were aggregated together with macrophages. AFB seen in lymph node sections showed at least some fragmentation.

Appendix V: Diagnostic information available for AR and BI ERS

TABLE 29: TOTAL DIAGNOSTIC INFORMATION AVAILABLE FOR BOTH ERS POPULATIONS

Island/Cohort	Clinical assessment			Serology			PCR		
	Total	Pos.	Neg.	Total	Pos.	Neg.	Total	Pos.	Neg.
BI									
Autumn 2016	26	7	19	25	9	16	1	1	NA
Spring 2017	26	9	17	26	10	16	15	7	8
Autumn 2017	20	4	16	20	5	15	11	5	6
Spring 2018	25	4	21	25	5	20	9	1	8
Autumn 2018	29	6	23	29	5	24	29	2	27
Carcasses*	18	7	11	NA	NA	NA	18	13	5
Total	144	37	107	125	34	91	83	29	54
AR									
Spring 2017	17	0	17	17	1	16	17	0	17
Autumn 2017	6	0	6	6	0	6	5	0	5
Spring 2018	14	0	14	14	0	14	9	0	9
Autumn 2018	25	0	25	25	0	25	25	2	23
Carcasses*	29	0	29	NA	Na	NA	29	3	26
Total	91	0	91	62	1	61	85	5	80

**On BI three ERS were seen live and included as carcasses as well (all leprosy cases), on AR four (one colonised).*